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Molekulární charakterizace gregarin flebotomů a jejich interakce s hostitelem

Sand fly gregarines: their molecular characterization and host-parasite interactions

Ph.D. Thesis / Disertační práce

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Praha 2011

I declare that most of the work presented in this thesis was accomplished by myself. This thesis was written by myself, all the literary sources were properly cited, and it has not been used as a final work towards any other university degree.

Prohlašuji, že naprostá většina výsledků prezentovaných v této práci byla dosažena mnou, že jsem disertační práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Prohlašuji, že naprostá většina výsledků prezentovaných v této práci byla dosažena Lucií Lantovou. Lucie se významně podílela na experimentální práci a na sepsání prezentovaných publikací.

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ABSTRACT

Gregarines (Apicomplexa: Gregarinasina) are monoxenous parasites of invertebrates. Those found in sand flies (Diptera: Psychodidae) and mosquitoes (Diptera: Culicidae) used to be considered a single eugregarine genus *Ascogregarina*. Our phylogenetic analyses of the gregarine SSU rDNA, including newly obtained sequences of three species from sand flies, showed that mosquito and sand fly gregarines are closely related to neogregarines, and most importantly, they form two disparate monophyletic groups. Based on these molecular features, accompanied by biological differences, we established a new genus *Psychodiella* for the gregarines from sand flies, reserving the genus *Ascogregarina* for the mosquito gregarines.

In the new genus, two new species *Psychodiella sergenti* from *Phlebotomus sergenti* and *Psychodiella tobbi* from *Phlebotomus tobbi* were described. They differ in the life cycles (sexual development of *Ps. sergenti* is triggered by a blood meal intake) and morphology of their life stages, mainly oocysts. The susceptibility of five sand fly species to both gregarines showed their strict host specificity, as they were able to fully develop and complete the life cycle only in their natural hosts.

The life cycle of *Ps. sergenti* was studied in detail using various microscopical methods. Oocysts are attached to the chorion of sand fly eggs. Sporozoites, with a three-layered pellicle and mucron, attach to the 1st instar larval intestine but are never located intracellularly. In the 4th instar larvae, the gregarines occur in the ectoperitrophic space and later in the intestinal lumen. In adults, the parasites appear in the body cavity, and the sexual development of *Ps. sergenti* takes place only in blood-fed females; gametocysts attach to the accessory glands and oocysts are injected into their lumen.

Psychodiella sergenti was proven to have a negative impact on its host; the infection significantly decreases the survival of various sand fly stages; however, it has no negative effect on the blood-fed female fecundity and mortality. A tenfold increase in the infection dose (5 vs. 50 gregarine oocysts per one sand fly egg) leads to roughly a tenfold, twofold and threefold increase in the number of gamonts in the 4th instar larvae, in females and males, respectively.

Even though sand fly gregarines are pathogenic parasites with interesting biology, they are not given the attention they deserve. This work attempts to fill in the missing information by giving a comprehensive insight into the sand fly gregarine taxonomy, host specificity, life cycle and pathogenicity.

ABSTRAKT

Gregariny (Apicomplexa: Gregarinasina) jsou parazité bezobratlých živočichů. Druhy popsané z flebotomů (Diptera: Psychodidae) a komárů (Diptera: Culicidae) byly původně řazeny mezi eugregariny rodu *Ascogregarina*. Sekvenací SSU rDNA tří druhů gregarin z flebotomů a jejich srovnáním s dostupnými sekvencemi ostatních druhů gregarin jsme však prokázali, že komáří i flebotomí gregariny jsou mnohem bližší neogregarinám a tvoří dvě oddělené skupiny. Protože toto zjištění je podpořeno i odlišnými biologickými vlastnostmi, rozdělili jsme rod *Ascogregarina* na dva a gregariny z flebotomů byly zařazeny do nového rodu *Psychodiella*.

V rámci tohoto nového rodu jsme popsali dva nové druhy: *Psychodiella sergenti* z *Phlebotomus sergenti* a *Psychodiella tobbi* z *Phlebotomus tobbi*. Tyto druhy se liší v životním cyklu (sexuální vývoj *Ps. sergenti* je v dospělcích podmíněn sáním krve) a velikostí i morfologií životních stádií, především oocyst. Dále jsme prokázali vysokou hostitelskou specifitu těchto gregarin; při pokusných infekcích pěti druhů flebotomů se *Ps. sergenti* a *Ps. tobbi* plně vyvíjely pouze ve svých přirozených hostitelích.

Životní cyklus *Ps. sergenti* byl detailně prostudován za použití různých mikroskopických metod. Oocysty parazita jsou přichyceny na chorion vajíček flebotomů. V larvách 1. instaru se sporozoiti s třívrstevnou pelikulou a mukronem vyskytují v ektoperitrofickém prostoru, někdy přichycení k epiteliálním buňkám, avšak nikdy nebyli lokalizováni intracelulárně. V larvách 4. instaru se gregariny nacházejí v ektoperitrofickém prostoru střeva a u starších jedinců v jeho lumen. V dospělcích pak gregariny osidlují tělní dutinu a v samicích po sání krve dochází k sexuálnímu vývoji, kdy se gametocysty přichycují k přídatným žlázám a oocysty jsou injikovány do jejich lumen.

Dále jsme prokázali, že *Ps. sergenti* má negativní vliv na svého hostitele; zvyšuje mortalitu nedospělých stádií a dospělých samců a samic flebotomů, nemá však vliv na fekunditu nebo mortalitu nasátých samic. Při použití desetkrát vyšší infekční dávky (5 vs. 50 oocysts na vejce) došlo k přibližně desetinásobnému zvýšení počtu gregarin v larvách 4. instaru, zatímco v samicích a samcích bylo dvoj či trojnásobné.

I přesto, že gregariny flebotomů jsou patogenní pro své hostitele a mají velmi zajímavou biologii, nejsou častým předmětem studia. V této práci jsme se snažili doplnit chybějící informace o těchto parazitech a podáváme souhrnnou studii o jejich molekulární taxonomii, hostitelské specifitě, životním cyklu a patogenitě.

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1 OBJECTIVES

Gregarines (Apicomplexa: Gregarinasina) are monoxenous parasites usually present in the body cavity or digestive tract of a number of invertebrate hosts. Along with other pathogens and parasites, they can be also found in phlebotomine sand flies (Diptera: Psychodidae). The sand fly gregarines have been recorded from many phlebotomine species, their pathogenic effect was shown experimentally, and from direct experience, they can seriously harm laboratory-reared colonies. Nevertheless, information available about them is insufficient. Prior to the publication of the manuscripts presented in this thesis, there had been only three described species of sand fly gregarines. Furthermore, host specificity and pathogenicity of these parasites was evaluated experimentally in a single study, and closer descriptions of the life cycle and fine structure were available only about one species. On the other hand, gregarines from mosquitoes (Diptera: Culicidae) have been widely studied, and presently, there are nine described species and a great deal of information about their pathogenic effects and host specificity. Furthermore, the only two studies dealing with molecular phylogeny of ascogregarines is accomplished about them.

In 1998 and 2005, a colony of *Phlebotomus sergenti* Parrot 1917 and *Phlebotomus tobbi* Adler, Theodor and Lourie 1930 were established in our laboratory. After several generations, adults began to suffer from a high mortality rate, and dissections revealed their hemocoels heavily infected by gregarine gamonts and gametocysts. To reduce the intensity of infection and increase fitness of the colonies, eggs had to be washed by a series of reagents. However, this procedure never cleaned them completely and had to be repeated every generation. Because gregarines had never been recorded in *Ph. sergenti* and *Ph. tobbi*, and results of other studies showed their strict host specificity, we considered these two gregarines new species. During our research, we had the opportunity to compare them to the most studied sand fly gregarine, formerly known as *Ascogregarina chagasi* (Adler and Mayrink 1961) from *Lutzomyia longipalpis* (Lutz and Neiva 1912).

The main objectives of this thesis are listed below. Special attention was given to a gregarine from *Ph. sergenti*, an important vector of *Leishmania tropica* (Euglenozoa: Kinetoplastea).

- To reveal the taxonomic position of sand fly gregarines by means of sequencing and comparing their genes for SSU rRNA and their biological features.

- To characterize two new species of gregarines from sand flies *Ph. sergenti* and *Ph. tobbi* by evaluating their host specificity and comparing their morphology and life cycles.

- To describe the life cycle of the gregarine from *Ph. sergenti* in more detail using various microscopical methods.

- To evaluate effects of the gregarine from *Ph. sergenti* on its hosts.

2 INTRODUCTION

2.1 Phlebotomine sand flies and their pathogens

Phlebotomine sand flies are blood-sucking insects and important vectors of human pathogens. Over 700 species have been described, and two genera feed on blood of mammals transmitting *Leishmania* (Euglenozoa: Kinetoplastea), *Bartonella* (Proteobacteria) and phleboviruses: *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Lewis 1973; Lane 1993; reviewed by Sadlova 1999). While there are many studies on the biology of adult sand flies, information about their breeding sites and larval development is lacking (Feliciangeli 2004). The adults feed on plant sugars, and females need blood to acquire nutrients for egg production. Some species are capable of autogeny (production of the first batch of eggs without taking a blood meal) and some feed on blood more than once for each batch of eggs (reviewed by Killick-Kendrick 1999). The eggs are usually laid six to ten days post blood meal (Volf and Volfova 2011) to a moist soil, animal burrows, caves or leaf litter (Lane 1993). The 1st instar larvae hatch usually from six to ten-day old eggs, and larval development lasts around three weeks (Volf and Volfova 2011). Sand flies have four larval instars feeding on organic detritus (Lane 1993) and sometimes on dead bodies of adults (Adler and Mayrink 1961). The terrestrial development of the larvae in dark humid sites facilitates growth and persistence of various sand fly entomopathogens (Warburg et al. 1991). A short overview of these organisms follows.

Mites (Arthropoda: Acariformes) have been recorded from a number of sand fly species, and a comprehensive overview of these pathogens was given by Lewis and Macfarlane (1981). The most abundant families are Trombidiidae and Stigmaeidae (McConnell and Correa 1964; Martinez-Ortega et al. 1983; Reeves et al. 2008), which are usually attached to the exoskeleton of adult flies and their presence can be determined from visible scars (Martinez-Ortega et al. 1983).

Various nematodes have been described from sand flies. Some of them negatively affect their hosts and are considered useful for biological control. For example, members of the family Tylenchidae (Nematoda), found by McConnell and Correa (1964) or Warburg (1991), can sterilize female sand flies (Poinar et al. 1993).

Didilia ooglypta (Adenophorea: Tetradonematidae), described by Tang et al. (1997) from *Phlebotomus papatasi* and *Ph. sergenti*, negatively affects larval development, causes sterility of males (Pires et al. 1997) and increases the mortality of adult sand flies (Killick-Kendrick et al. 1989). Other nematode, probably from the family Steinernematidae, negatively affects survival and blood feeding of sand fly females (Secundio et al. 2002). *Mastophorus muris* (Secernetea: Spirocercidae) was recorded in sand flies according to Young and Lewis (1977) and by Killick-Kendrick et al. (1976), and other spirurid nematodes were found in *Lutzomyia townsendi* by Warburg (1991).

Saprophytic non-pathogenic fungi are common in the sand fly environment, where they are ingested by larvae, and they have been recorded from their intestines and also the body surface of adults (Warburg 1991). Furthermore, pathogenic fungi were observed from a number of sand fly species, and it has been suggested that they could be used in biological control replacing chemical insecticides (Amora et al. 2009). McConnell and Correa (1964) recorded fungi (Zygomycota: Entomophthorales, according to Warburg et al. (1991)) from ten *Lutzomyia* species. They are associated with reproductive organs and the body cavity, and they are suspected to disrupt normal physiology of females. Entomophthoralean fungi were also found in the body cavity of *Phlebotomus ariasi* (Rioux et al. 1966), in the thoracic muscles of *Lutzomyia pia* (Warburg 1991), and according to Warburg (1991), the vast number of fungal reports from sand flies reviewed by Young and Lewis (1977) could be Entomophthorales as well. Furthermore, these fungi occur in laboratory-reared colonies causing periodical increase in the adult sand fly mortality (Volf, personal communication). The fungal infection seems to be more harmful to adult sand flies than to the larvae, as *Beauveria bassiana* (Ascomycota: Hypocreales) decreases hatching rate (Amora et al. 2009) and adult survival and fecundity, while the larvae are not affected (Warburg 1991). The usability of this fungus in biological control is questioned by Reithinger et al. (1997); these authors showed that sand flies are not susceptible to *B. bassiana* infection in nature.

Microsporidia (Fungi) are obligatory intracellular parasites of eukaryotes. In sand flies, they have been recorded in a few species (reviewed by Young and Lewis 1977; 1980), and only two have been described: *Flabelliforma montana* (Pansporoblastina) from *Ph. ariasi* (Canning et al. 1991) and *Vavraia lutzomyiae* (Pansporoblastina) from *L. longipalpis* (Matos et al. 2006). The latter parasites are

located in the larval abdomen and sometimes in the Malpighian tubules and midgut of adults. Warburg et al. (1991) suggest that Microsporidia are much more abundant in sand flies than previously recorded.

The order Trypanosomatida (Euglenozoa: Kinetoplastea) includes one of the most important parasites transmitted by sand flies, *Leishmania*, a causative agent of human leishmaniasis. This disease has two basic clinical forms: cutaneous and visceral, and it occurs in tropical and subtropical areas of America, Africa, Europe and Asia (Rutledge and Gupta 2009). Apart from *Leishmania*, sand flies also transmit other trypanosomatids: *Endotrypanum* (reviewed by Shaw 1981), a parasite of sloths and *Trypanosoma* (reviewed by McConnell and Correa 1964; Williams 1976). Other kinetoplasteans found in sand flies are *Herpetomonas phlebotomi*, the genus *Crithidia* (Jenkins 1964) and a parasite resembling *Leptomonas* or *Phytomonas* (Warburg 1991).

Other pathogens found in sand flies are members of the phylum Apicomplexa. Sand flies are vectors of *Plasmodium* (Apicomplexa: Haemosporida), a causative agent of reptilian malaria (reviewed by Telford 1994). A coccidian parasite *Adelina riouxi* (Apicomplexa: Coccidiasina) was recorded in *Phlebotomus perniciosus* and *Sergentomyia minuta* (Rioux et al. 1972), and Warburg (1991) suggests that it could serve as a natural control agent of phlebotomine sand flies. Gregarines of the former genus *Ascogregarina* Ward, Levine and Craig 1982 have been recorded from a vast number of sand fly species; however, before the publication of our study (Lantova et al. 2010) presented in this thesis, only three gregarine species had been described. Chapter 2.2 brings detailed information about these undervalued parasites.

Bacteria are important pathogens occurring in sand flies. *Bartonella bacilliformis*, a causative agent of human disease called Oroya fever or verruga peruana in Peru, Ecuador and Colombia, is transmitted by *Lutzomyia* species (Rutledge and Gupta 2009). A number of bacteria have been recorded from sand flies in the field and from the laboratory: *Spirochaeta* (=*Treponema*) *phlebotomi* (Spirochaetae) (Young and Lewis 1977), *Pseudomonas* (Proteobacteria), *Rickettsia* (Proteobacteria) (Young and Lewis 1977; Warburg 1991; Reeves et al. 2008) and Gram-negative Enterobacteriacae (Proteobacteria) and non-Enterobacteriacae from wild-caught sand flies (Oliveira et al. 2000; Gouveia et al. 2008; Hillesland et al. 2008). The microbial

community in the gut of pre-imaginal stages and adults is different (Volf et al. 2002; Guernaoui et al. 2011); however, some bacteria can be transmitted transstadially from larvae to the adults (Volf et al. 2002). Furthermore, blood feeding causes a temporary increase in the bacterial counts in females (Volf et al. 2002).

Serratia marcescens (Proteobacteria) commonly appears in sand fly colonies and in wild-caught sand flies (Gouveia et al. 2008). Generally, it is pathogenic to insects (Seitz et al. 1987; Lauzon et al. 2003); however, it was not shown to affect sand flies (Warburg 1991). On the other hand, *Bacillus thuringiensis* var. *israelensis* (Firmicutes) toxin (used in biological control of insects) causes higher mortality of sand flies (Yuval and Warburg 1989), and *Bacillus sphaericus* was shown to negatively affect the survival of sand fly larvae (Pener and Wilamowski 1996; Robert et al. 1997). *Wolbachia* (Proteobacteria) infections have been recoded from a number of Old and New World sand flies from nature and colonies (Ono et al. 2001). They are maternally transmitted, found in arthropods and nematodes, and they are known to affect their reproduction (reviewed by Soares and Turco 2003).

Phlebotomine sand flies are also vectors of three human important genera of phleboviruses: *Phlebovirus* (Bunyaviridae), *Vesiculovirus* (Rhabdoviridae) and *Orbivirus* (Reoviridae) (reviewed by Depaquit et al. 2010). There are 38 distinct *Phlebovirus* serotypes, 66% of which are transmitted by sand flies, and they have been isolated in America, central Asia, Africa and southern Europe. Eight phleboviruses (including e.g. Naples, Punta Toro, Sicilian and Toscana) are the causative agents of sand fly fever of humans (Tesh 1988); however, effects of these viruses on their vectors are not known (Warburg et al. 1991).

Entomopathogenic cytoplasmic polyhedrosis viruses CPVs (Reoviridae) have been isolated from naturally infected colonies of *Ph. papatasi* (Warburg and Ostrovska 1987) and *L. longipalpis* (Warburg and Pimenta 1995). Despite the fact that CPVs do not affect adult sand flies (Warburg and Ostrovska 1987), they decrease the vectorial capacity and competence of *L. longipalpis* to *Leishmania donovani chagasi* (Warburg and Pimenta 1995) and *Ph. papatasi* to *Leishmania major* by disrupting the midgut epithelium and peritrophic matrix (Warburg and Ostrovska 1987). Therefore, CPVs were considered useful in biological control of leishmaniasis. However, differences in the susceptibility of sand flies to CPVs (Warburg 1991) and their relatively low pathogenicity to adults are making them less suitable (Warburg et al. 1991).

2.2 Gregarines

2.2.1 Gregarine taxonomy

Gregarines, class Conoidasida, subclass Gregarinasina are members of the phylum Apicomplexa, a large and diverse group of parasites belonging to Alveolata. They are thought to be the earliest lineage of apicomplexans, phylogenetically very close to members of the genus *Cryptosporidium* (Apicomplexa: Coccidiasina) (Carreno a et al. 1999; Leander et al. 2003a; Leander and Keeling 2004; Rueckert and Leander 2009; Templeton et al. 2009). The "sister" relationship of gregarines and cryptosporidians is supported by some aspects of their morphology and life cycle; both are monoxenous parasites, and both have specialized organelles of attachment (a mucron or epimerite in the gregarines and a feeder organelle in the cryptosporidians), with resembling attachment sites (Valigurova et al. 2007). Furthermore, in both of these genera, the presence of an apicoplast is disputable; it was not proven either in the gregarines (Obornik et al. 2002; Toso and Omoto 2007) or in the cryptosporidians (Zhu et al. 2000).

Gregarines used to be divided into two groups depending on the presence or absence of merogony, an asexual replication: Eugregarinida without merogony and Schizogregarinida with merogony in their life cycle. However, Grasse (1953) divided the latter into archigregarines ("primitive" life cycle with merogony) and neogregarines (septate eugregarines with secondarily reacquired merogony). Currently (according to Perkins et al. 2000), there are three orders: Archigregarinorida (parasites of annelids, sipunculids, hemichordates and ascidians, with merogony), Neogregarinorida (found in arthropods, mostly dipterans, with merogony) and Eugregarinorida (found mostly in annelids and arthropods, without merogony).

Based on their morphology, trophozoites of gregarines can be divided into two major groups (Schrevel and Philippe 1993); aseptate (monocystid or acephaline) that are not divided into regions except the anterior part of the cell, mucron, serving as the attachment apparatus. These gregarines are members of archigregarines and the suborder Blastogregarinorina and Aseptatorina within eugregarines (Perkins et al. 2000). The other group, cephaline (septate or polycystid) gregarines, with the cell divided into segments by septa, are members of the eugregarine suborder Septatorina (Perkins et al. 2000). The cephaline tricystid gregarines have epimerite for attachment to the host cell, protomerite and deuteromerite containing nucleus. The cells of

polysegmented gregarines are divided into several sections (Schrevel and Philippe 1993).

Leander et al. (2003b; 2006) points out that, considering the behaviour and the morphology, trophozoites are the most distinctive, abundant and sufficiently variable stages in the gregarine life cycle, and they should be used in the gregarine taxonomy. However, gregarine taxonomy based solely on the morphology and life cycle can be problematic, due to many species and genera not having sufficient descriptions or not being characterized by clear and unique features, as remarked by Clopton (2009). For example, members of the family Gigaductidae had been originally considered eugregarines, because they possess some eugregarine features such as protomerite and deuteromerite, characteristic epimerite and the type of gametocyst dehiscence. However, after Tuzet and Ormieres (1966), Ormieres (1971) and Massot and Ormieres (1979) showed that members of this group undergo merogony in their coleopteran and orthopteran hosts, the family Gigaductidae was transferred into Neogregarinorida. Another example of a challenging gregarine taxonomy is the order Archigregarinorida and the phylogenetic position of the family Selenidiidae. Levine (1971) emphasize that not all the selenidiid species have been described as having merogony in their life cycle, and therefore, he divided them into two families, one (with merogony) within archigregarines and one (without merogony) within eugregarines. This arrangement was accepted by Perkins et al. (2000). Contrastingly, Schrevel and Philippe (1993) find this proposal unacceptable, because the absence of merogony in some of the species is insufficient character for them to be included within eugregarines, and Schrevel (1971) characterizes archigregarines as intestinal parasites of polychaete worms, with ultrastructural organisation of the trophozoites similar to that of dissemination forms and with a well-defined pellicular fibrillar system.

To clarify the relationships within gregarines, molecular phylogenetic methods have been used, particularly the sequencing and phylogeny of genes, mostly for SSU rRNA. Based on the sequences of SSU rDNA and β-tubulin, aseptate eugregarines and neogregarines are very closely related (Carreno et al. 1999; Leander et al. 2003a; 2003b; 2006), and because neogregarines combine features of both coccidia and eugregarines, they could be the link between these two groups (Vavra and McLaughlin 1970).

2.2.2 Gregarine life cycles and characteristics

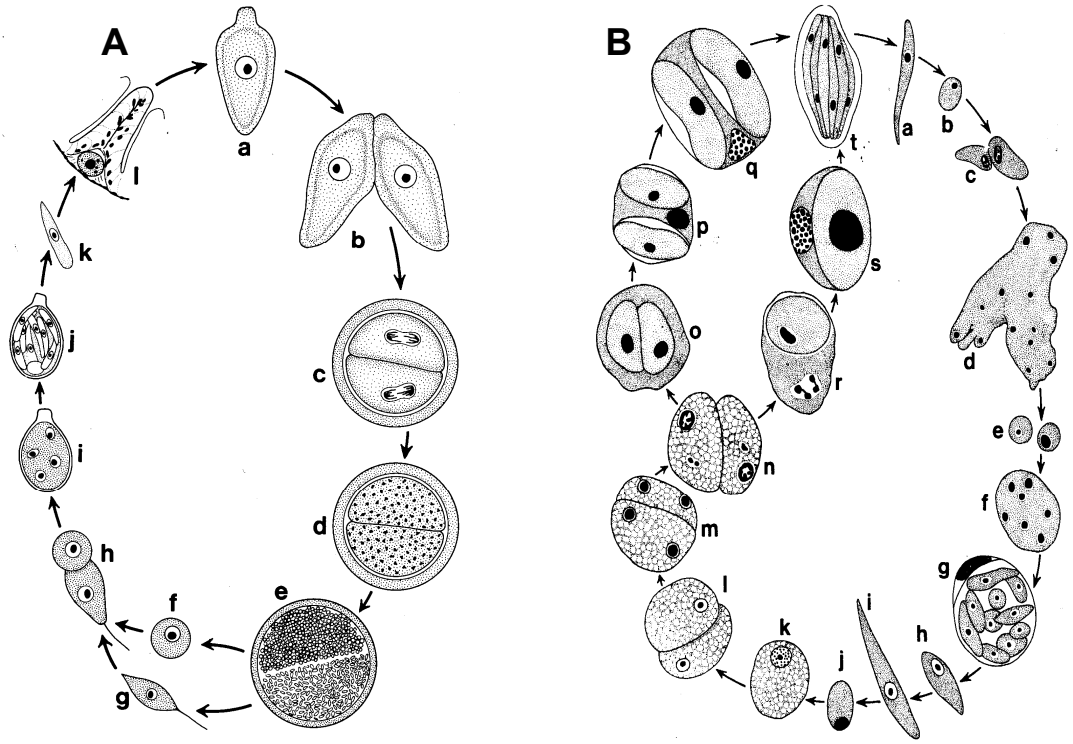
Gregarines can be found usually in the body cavity or digestive tract of a number of invertebrate hosts, namely annelids, molluscs, nemerteans, echinoderms, urochordates and arthropods (reviewed by Schrevel and Philippe 1993). The gregarine life cycle can be summarized as follows: the infective stages are oocysts containing sporozoites. After dehiscence of the oocysts, sporozoites are released, and they develop either attached to the host epithelium or intracellularly into trophozoites. Detachment of the trophozoites from the host cell is followed by the sexual phase of the life cycle, beginning with association of two mature gamonts into syzygy. The two gamonts in the syzygy are then enclosed in a cyst wall, and gametocyst is formed. Within the gametocyst, during gamogony, each gamont undergoes multiple nuclear divisions without cytokinesis, leading to production of gametes. After fertilization, during sporogony, zygotes differentiate by mitoses into oocysts with sporozoites. These can be released from the gametocysts by various ways, mostly by a simple rupture of the gametocyst or through a sporoduct (reviewed by Schrevel and Philippe 1993).

Order Eugregarinorida comprises gregarines with similar life cycle to that described above; the life cycle of *Lecudina* sp. (Eugregarinorida: Aseptatorina), a parasite of *Nereis diversicolor* (Annelida: Polychaeta) is shown in Fig. 1A. On the other hand, the life cycle of neogregarines (and some archigregarines) includes merogony, a multiple asexual fission, which causes destruction of host tissues and fast spread of the parasites within the host. The life cycle of *Mattesia dispora* (Neogregarinorida: Lipotrophidae), a parasite of flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae), consists of two merogonies (micronuclear and macronuclear) and is shown in Fig. 1B.

All apicomplexans are characterized by the presence of a set of anterior structures for attachment, the apical complex (Adl et al. 2005), which consists of polar rings, rhoptries, micronemes, conoid and subpellicular microtubules and is important in the host-cell invasion (reviewed by Dubremetz et al. 1998). Other characteristic apicomplexan feature is a three-layered pellicle formed from flattened subpellicular vesicles (alveoli) and the plasma membrane (Adl et al. 2005). The surface of eugregarines is associated with 12 nm filaments, an internal lamina and apical rippled dense structures (Schrevel et al. 1983). The pellicle of trophozoites can be organized into folds; archigregarines have large longitudinal folds (e.g. Schrevel 1971), neogregarines do not possess any folds (e.g. Vavra and McLaughlin 1970), polycystid eugregarines have mostly narrow longitudinal folds (e.g. Schrevel et al. 1983), and

monocystid gregarines are more variable, with some having typical longitudinal folds and some more complex ones (reviewed by Schrevel and Philippe 1993).

Fig. 1. The life cycle of *Lecudina* sp. (Eugregarinorida) (A) and *Mattesia dispora* (Neogregarinorida) (B).



***Lecudina* sp.** (a) Gamont in the intestinal lumen; (b) syzygy; (c) gametocyst; (d, e) gamogony and formation of gametes; (f) female gamete; (g) male gamete; (h) fertilization; (i) young oocyst; (j) mature oocyst with sporozoites; (k) sporozoite; (l) young trophozoite in the intestinal epithelium of the host. Based on Perkins et al. (2000).

Mattesia dispora (a) Sporozoite in the host intestinal lumen; (b – e) first (micronuclear) merogony in the fat body; (f, g) second (macronuclear) merogony in the fat body; (h – k) formation of gamonts; (l – n) syzygy; (o – q) sporogony of a dispore form; (r, s) sporogony of a monospore form; (t) mature oocyst containing sporozoites. Based on Perkins et al. (2000).

Trophozoites of most gregarines are motile. Gliding, the most studied type of movement, is without any change of cell shape and is always accompanied by the formation of a mucus trail (Mackenzie and Walker 1983), which serves as a lubricant (Schrevel et al. 1983). Gliding is driven by actin-myozin interactions (reviewed by King 1988; Heintzelman 2003). Another type of movement, pendular or rolling, was

described in selenidiid gregarines, and peristaltic movement was recorded in some species of eugregarines (reviewed by Schrevel and Philippe 1993).

Apart from the common cytoplasmic organelles such as nucleus, mitochondria and Golgi apparatus, which can differ among gregarine species, there are also various inclusions including lipid droplets and spherical or ovoid bodies of storage polysaccharide, sometimes called paraglycogen. The paraglycogen granules are of the amylopectin type (Schrevel 1970; Mercier et al. 1973). They resemble the amylopectin found in *Eimeria* (Apicomplexa: Coccidiasina) species and consist of about 20 glucose residues. Structurally, they are intermediate between a plant amylopectin and an animal glycogen similar to sweet corn phytoglycogen (Mercier et al. 1973). Because of their similarity to glycogen, they are strongly PAS (periodic acid-Schiff)-positive, and therefore Jennings (1961) suggested using PAS reaction to demonstrate gregarines and coccidia in the host tissues.

2.3 Gregarines parasitizing mosquitoes and sand flies

The present thesis deals with the life history, pathogenic effects, host specificity and taxonomy of mosquito and sand fly gregarines bringing new data and also introducing changes in their systematics. However, the following overview of published information about sand fly gregarine host-parasite interactions and systematics represents the situation before studies of our team by Votypka et al. (2009), Lantova et al. (2010) and Lantova et al. (2011a; 2011b) were published, accepted for publication or submitted. The new data and systematic arrangement is presented in the result, summary and conclusion sections (see chapter 3, 4 and 5).

2.3.1 Taxonomy of mosquito and sand fly gregarines

Members of the genus *Ascogregarina* (syn. *Monocystis* von Stein 1848, *Lankesteria* Mingazzini 1891 and *Ascocystis* Grasse 1953) are aseptate eugregarines (Eugregarinorida: Aseptatorina) of the family Lecudinidae. Out of 16 named species of the genus (Perkins et al. 2000), three had been described from sand flies (before 2010) and nine from mosquitoes (Table 1). The terminology and history of the final designation of mosquito and sand fly gregarines is complex. The type species of the genus is *Ascogregarina culicis* (Ross 1898), the first mosquito gregarine originally

described as *Gregarina culicis* by Ross (1898) and renamed as *Lankesteria culicis* by Wenyon (1911). The first described sand fly gregarine was originally named *Monocystis mackiei* by Shortt and Swaminath (1927) or *Lankesteria phlebotomi mackiei* by Missiroli (1932) and later renamed as *Ascogregarina mackiei* (Shortt and Swaminath 1927). Grasse (1953) proposed a new name *Ascocystis* for gregarines of the genus *Lankesteria* parasitizing insects, and he renamed *Lankesteria culicis* as *Ascocystis culicis*. Similarly, Ormieres (1965) and Tuzet and Rioux (1966) renamed *L. phlebotomi mackiei* as *Ascocystis mackiei*, and later, Scorza and Carnevali (1981) brought morphological evidence placing sand fly gregarines of the genus *Monocystis* into the genus *Ascocystis*. Ormieres (1965) and Levine (1977) accepted *Ascocystis* from Grasse (1953) for parasites of Diptera and restricted *Lankesteria* to parasites of ascidians. However, the name *Ascocystis* is a synonym for *Ascocystis* Bather 1889 used for fossil crinoid echinoderm, and therefore Ward et al. (1982) established a new name *Ascogregarina* for the gregarines from Diptera formerly known as *Ascocystis*.

Even though there were numerous studies about ascogregarines, mostly those from mosquitoes, dealing with their effects on hosts, morphology, life cycle, prevalence and host specificity, information about their molecular taxonomy was lacking. Up until 2009, genes for SSU rRNA had been sequenced for *Ascogregarina armigerei* (Lien and Levine 1980), *Ascogregarina* sp. from *Ochlerotatus japonicus*, *As. culicis* and *Ascogregarina taiwanensis* (Lien and Levine 1980) by Roychoudhury et al. (2007a). Partial sequences of SSU rDNA and 28S rDNA and sequences of ITS1, 5.8S rDNA and ITS2 of *Ascogregarina barretti* (Vavra 1969), *As. culicis* and *As. taiwanensis* were submitted by Morales et al. (2005). Furthermore, there were directly submitted sequences of actin gene, partial SSU rDNA and ITS1, 5.8S rDNA, ITS2, 26S rDNA and 5S rDNA of *As. taiwanensis*. There was only one study dealing with the phylogenetic position of mosquito ascogregarines by Roychoudhury et al. (2007a), who sequenced SSU rDNA of four ascogregarine species showing their monophyletic position with other gregarines and close relationship with cryptosporidians. More recently, a whole-genome-sequence survey for *As. taiwanensis* was accomplished by Templeton et al. (2010).

Table 1. A list of designated sand fly and mosquito gregarines of the genus *Ascogregarina* before 2009.

name	original name	host species
Ascogregarina culicis (Ross 1898)	*Gregarina culicis*	***Aedes aegypti*** (Lin. 1762)
As. tripteroidesi (Bhatia 1938)	*Lankesteria tripteroidesi*	***Tripteroides dofleini*** (Guenther 1913)
As. barretti (Vavra 1969)	*Lankesteria barretti*	***Ae. triseriatus*** (Say 1823)
As. clarki (Sanders and Poinar 1973)	*Lankesteria clarki*	***Ae. sierrensis*** (Ludlow 1905)
As. armigerei (Lien and Levine 1980)	*Ascocystis armigerei*	***Armigeres subalbatus*** (Coquillett 1898)
As. lanyuensis (Lien and Levine 1980)	*Ascocystis lanyuensis*	***Ae. alcasidi*** Huang 1972
As. taiwanensis (Lien and Levine 1980)	*Ascocystis taiwanensis*	***Ae. albopictus*** (Skuse 1894)
As. geniculati Munstermann and Levine 1983	*Ascogregarina geniculati*	***Ae. geniculatus*** (Olivier 1791)
As. polynesiensis Levine 1985	*Ascogregarina polynesiensis*	***Ae. polynesiensis*** Marks 1951
As. mackiei (Shortt and Swaminath 1927)	*Monocystis mackiei*	***Phlebotomus argentipes*** Ann. and Brun. 1908
As. chagasi (Adler and Mayrink 1961)	*Monocystis chagasi*	***Lutzomyia longipalpis*** (Lutz and Neiva 1912)
As. saraviae Ostrovska, Warburg and Montoya-Lerma 1990	*Ascogregarina saraviae*	***L. lichyi*** (Floch and Abonnenc 1950)

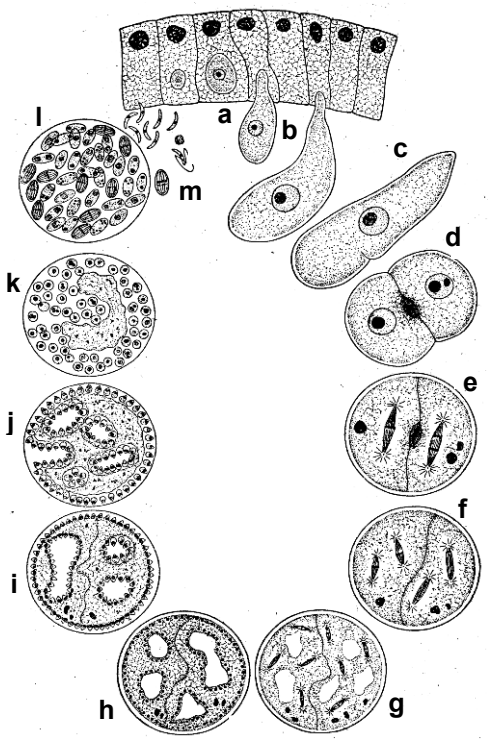
2.3.2 Gregarines from mosquitoes and their life cycles

The life cycle of *As. culicis* (Fig. 2), the type species of the genus *Ascogregarina* is very similar to other mosquito ascogregarines and is used as a typical example of a mosquito gregarine life cycle (minor interspecific differences and details are explained for each species separately).

The mosquito larvae become infected by ingesting *Ascogregarina* oocysts. Each spindle-shaped oocyst contains eight sporozoites, which are released in the intestine and invade the epithelial cells. Inside the cells, they develop into trophozoites, and later, when the epithelial cell ruptures, these are released into the gut lumen. During pupation, the gregarines migrate to the Malpighian tubules, where the sexual development takes

place; gamonts pair in syzygies and develop into gametocysts with oocysts inside. The oocysts are released during defecation with faeces into the water and infect newly hatched larvae. (Wenyon 1911; Walsh and Callaway 1969; McCray et al. 1970).

Fig. 2. The life cycle of *Ascogregarina culicis*. Life cycle stages: (a) intracellular trophozoite; (b) extracellular trophozoites; (c) gamont; (d) syzygy; (e – j) gamogony; (k) fertilization; (l) formation of oocysts; (m) oocysts with sporozoites. Location: (m, a, b) larval gut; (c – l) the Malpighian tubules of adults. Based on Lankester (1953).



***Ascogregarina culicis* (Ross 1898)** was described in India by Ross (1895) as *Gregarina culicidis* and later as *Gregarina culicis* (Ross 1898). Oocysts of this parasite are infective to all larval stages of *Aedes aegypti* (Lin. 1762); when the early larval instars are infected with the gregarine oocysts, the development of both the parasite and the host are synchronized, while the gregarine development stops at the gamont stage, when the oocysts are ingested by the late 4th instar larvae. Similar results were observed also for *As. taiwanensis* in *Aedes albopictus* (Skuse 1894) (Roychoudhury and Kobayashi 2006).

McCray et al. (1970) recorded sporozoites and trophozoites of *As. culicis* mostly in the anterior part of the larval midgut, while Walsh and Callaway (1969) in the posterior part. The sporozoites are 9.5 – 10 μm long with a tapered posterior part. Their

pellicle, according to the authors (Sheffield et al. 1971), consists of an outer and a thicker inner membrane. The anterior part of the sporozoite contains conoid, two apical rings and a polar ring. A "flask-shaped" organelle, observed in the anterior part of mostly extracellular sporozoites, was suggested to have a function in the host cell invasion (Sheffield et al. 1971). Trophozoites of *As. culicis* are 170 μm long, gametocysts are 71 – 125 μm in diameter and oocysts are 11 μm long and 5 μm wide (Lien and Levine 1980).

Several studies dealing with the prevalence and seasonality of *As. culicis* in natural *Ae. aegypti* populations showed that e.g. in temperate Argentina, it is seasonally and spatially heterogeneous (Vezzani and Wisnivesky 2006; Albicocco and Vezzani 2009), and the prevalence of the parasite was 21.1% and 16.7%, respectively. One of the first country-wide surveys of *As. culicis* prevalence was given in Trinidad (Beier et al. 1995).

Ascogregarina tripteroidesi **(Bhatia 1938)** was found in *Tripteroides dofleini* (Guenther 1913) in Sri Lanka by Guenther (1914) and later denominated by Bhatia (1938). The whole life cycle was not described; the only known stages are trophozoites, which were recorded in the body cavity, trachea and anal gills of larvae.

Ascogregarina barretti **(Vavra 1969)** was described from *Aedes triseriatus* (Say 1823) in Texas by Vavra (1969). Trophozoites develop in the epithelial cells of the larval intestine, after reaching the size of 150 – 200 μm, they are released from ruptured cells and appear in the ectoperitrophic space of the intestine as gamonts. These grow up to the length of 310 μm, and during pupation, they enter into the Malpighian tubules. Gametocysts are 60 – 100 μm in diameter, and oocysts with eight sporozoites measure 11 × 5.4 – 5.7 μm. This gregarine differs from *As. culicis* in several features: the location of the trophozoites within the epithelial cells, position of their nucleus, character of longitudinal folds, the presence of a mucron and size of paraglycogen granules (Vavra 1969).

Ascogregarina clarki **(Sanders and Poinar 1973)** was described from *Aedes sierrensis* (Ludlow 1905) in California by Sanders and Poinar (1973). Several features distinguish this gregarine from *As. culicis* and *As. barretti*: the character of gamonts and their nucleus, structure and position of residual bodies in the oocysts, and the fact that the

trophozoites are always intracellular in the anterior part of the larval midgut. Sporozoites measure 8 × 1 – 2 μm, mature trophozoites 129.1 × 26 μm, and they have unresolved number of membranes in the pellicle. According to the authors, the passage of the trophozoites to the Malpighian tubules is rather passive, as the release of the gregarines from the epithelial cells is a result of natural histolysis of the midgut cells during pupation. Gamonts measure 226 × 31 μm, and gametocysts are 78 μm in diameter on average (Sanders a Poinar 1973).

Ascogregarina armigerei **(Lien and Levine 1980)** was described from *Armigeres subalbatus* (Coquillett 1898) in Taiwan. Trophozoites measure 135 × 23 μm and are contractile, gametocysts are 71.2 μm in diameter. The character and size of the oocysts (14.5 × 6 μm) clearly distinguishes between *As. culicis*, *As. armigerei*, *Ascogregarina lanyuensis* (Lien and Levine 1980) and *As. taiwanensis* (Lien and Levine 1980).

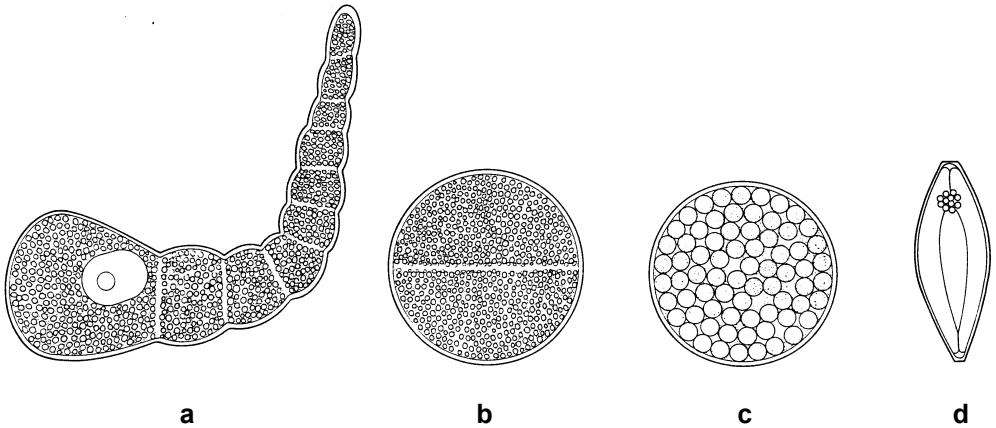
Ascogregarina lanyuensis **(Lien and Levine 1980)** was described from *Aedes alcasidi* Huang 1972 in Taiwan along with two other ascogregarine species (Lien and Levine 1980). Trophozoites measure 190 × 26 μm, gametocysts are 89.9 μm in diameter and oocysts are 9 μm long and 5 μm wide.

Ascogregarina taiwanensis **(Lien and Levine 1980)** was described from *Ae. albopictus* in Taiwan, and it is the most studied mosquito ascogregarine. The development of *As. taiwanensis* is influenced by the larval age at the time of infection in a similar pattern as in *As. culicis* (see above; Roychoudhury and Kobayashi 2006). Sporozoites invade the anterior third of the larval midgut epithelium; Chen et al. (1997a) usually found two of them in each cell. They are slender with a three-layered pellicle (recorded also in trophozoites) and possess typical apical complex with a conoid, polar rings, rhoptries, subpellicular microtubules and micronemes (Chen et al. 1997b). The amylopectin granules recorded in the oocysts disappear during the morphogenesis of the sporozoites (Chen et al. 1997b). The release of the sporozoites from the oocysts may be triggered by V-ATPase modulated alkalization in the anterior midgut of the mosquito, and V-ATPase may also play a role in the parasite invasion and formation of extracellular stages (Huang et al. 2006).

The development of the extracellular trophozoites is conditioned by their migration to the Malpighian tubules; the ones who fail to migrate undergo apoptosis (Fan-Chiang and Chen 2002). Actin and myosin are involved in this migration, and at the anterior end of the migrating parasite, a "protruding apparatus" with enhanced actin expression is formed (Chen and Fan-Chiang 2001). The sexual reproduction of *As. taiwanensis* in the Malpighian tubules is influenced and, to a certain level, synchronized with the host metamorphosis; increase of the level of the moulting hormone 20-hydroxyecdysone signals migration of the parasite and expedites the formation of gametocysts (Chen and Yang 1996; Chen 1999). Gamonts are 234 μm long, gametocysts are 87,5 μm in diameter (Lien and Levine 1980). Oocysts measure 10 × 5 μm according to Lien and Levine (1980) and 8.72 × 4.97 μm according to Chen et al. (1997b).

Ascogregarina geniculati **Munstermann and Levine 1983** was found in *Aedes geniculatus* (Olivier 1791) by Ganapati and Tate (1949) in England and by Kramar (1952) in the former Czechoslovakia, and it was originally described as *As. culicis*. However, Munstermann a Levine (1983), who were studying this gregarine in Sardinia, determined it as *As. geniculati* (Fig. 3). The main characteristic that can differentiate this species from other mosquito ascogregarines is the dimension of oocysts that measure 13.5 × 5 μm. Gamonts measure on average 175 × 31 μm and gametocysts are 77 μm in diameter (Munstermann and Levine 1983).

Fig. 3. ***Ascogregarina geniculati*** **life stages.** (a) Gamont; (b) young gametocyst containing macro- and microgametes; (c) gametocyst containing zygotes; (d) oocyst. Based on Munstermann and Levine (1983).



***Ascogregarina polynesiensis* Levine 1985** was found in *Aedes polynesiensis* Marks 1951 in Samoa by Pillai et al. (1976) and was, similarly to *As. geniculati*, originally described as *As. culicis*. However, Levine (1985) pointed out that *As. culicis* differs from the gregarine from *Ae. polynesiensis* and that Pillai et al. (1976) examined 325 *Ae. aegypti* (the natural host of *As. culicis*) and found only a single infected one, while the prevalence in *Ae. polynesiensis* was 39.5 %. Therefore, this gregarine was renamed to *Ascogregarina polynesiensis* (Levine 1985). Trophozoites have a mean dimension of 65 × 35 µm, gametocysts measure approximately 40 µm and the oocysts measure 9.32 × 4.24 µm (Pillai et al. 1976).

Other mosquito gregarines. According to some authors, *As. culicis* was found also in other mosquito species than *Ae. aegypti*: Kramar (1953) and Ganapati and Tate (1949) found it in *Ae. geniculatus*, Ray (1933) in *Ae. albopictus*, Feng (1930) in *Aedes koreicus* and Pillai et al. (1976) in *Ae. polynesiensis*. Vavra (1969) pointed out that possibly not all the descriptions of gregarines from mosquitoes were dealing with *As. culicis* as originally presumed, and he suggested that only the ones from *Ae. albopictus* and *Ae. koreicus* could be *As. culicis*. Munstermann and Levine (1983) consider the two gregarines from *Ae. geniculatus* to be *As. geniculati*, and Levine (1985) designated the gregarine from *Ae. polynesiensis* as *As. polynesiensis.*

A gregarine similar to *As. barretti* was found in Indiana in *Aedes hendersoni* (Rowton et al. 1987); however, the differences in the number of gregarine stages, location of the trophozoites within the larval gut and the presence of dead gamonts in cross-infections with *As. barretti* and *Ae. triseriatus* suggest that the gregarine from *Ae. hendersoni* is a new species, distinct from *As. barretti*. This was accepted by Chen (1999). Roychoudhury et al. (2007b) described ascogregarine from *O. japonicus* in Japan and consider it a new species *Ascogregarina* sp.

Other ascogregarines have been recorded from different mosquito species in USA, Brazil, West Africa, China, Malaysia, Philippines, Italy or France, (reviewed by Christophers 1960; Clark 1980), and Tuzet and Rioux (1966) gave an overview of gregarines from culicid, ceratopogonid, simuliid and psychodid Diptera comprising not only several eugregarine species, but also some neogregarines including *Caulleryella* (Neogregarinorida: Caulleryellidae) sp. This neogregarine is an intestinal extracellular parasite that has been described from several mosquito species (reviewed by Weiser 1966).

2.3.3 Gregarines from sand flies and their life cycles

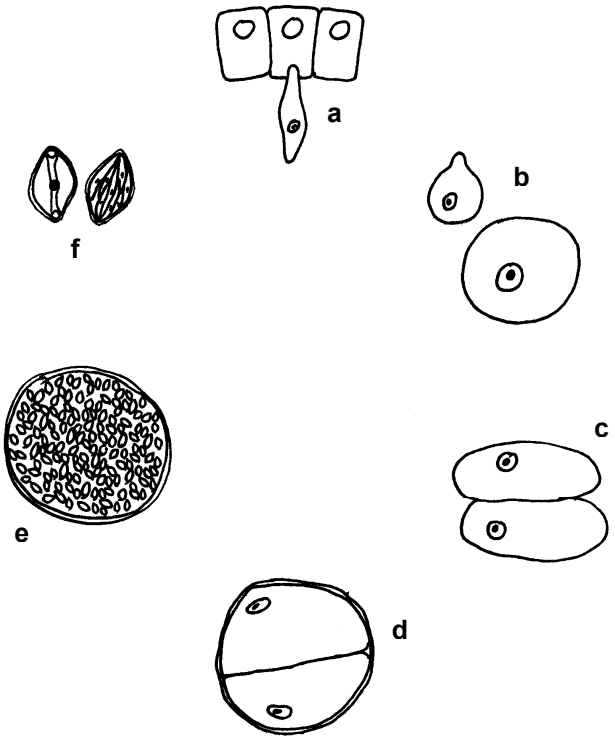
The life cycle of *As. chagasi* (Adler and Mayrink 1961) (see Adler and Mayrink 1961; Coelho and Falcao 1964; Warburg and Ostrovska 1991) is used as a typical example (details and interspecific differences are explained for each species separately). As there is no drawing of the life cycle of this gregarine in the literature, I summarized it in Fig. 4.

The first instar larvae are infected by swallowing spindle-shaped oocysts. Eight sporozoites released from these oocyst reside in the larval midgut, attach to the epithelial cells and develop into trophozoites. Later, gamonts can be found mostly in the larval gut lumen, where the gregarines undergo sexual development from the formation of syzygies to the production of oocysts. In adults, the gregarines are located in the body cavity forming syzygies and gametocysts with oocysts inside. The gametocysts attach to the accessory glands of females, and the oocysts are injected into their lumen. This was recorded by several authors (Adler and Mayrink 1961; Coelho and Falcao 1964; Lewis et al. 1970; Scorza and Carnevali 1981), and it is a unique mechanism of vertical transmission supporting the hypothesis about co-evolution of gregarines and sand flies (Ostrovska et al. 1990). During oviposition, contents of the glands including the oocysts are attached to the chorion of eggs and serve as a source of infection for newly hatched larvae. This general life cycle is modified in *As. mackiei*, where the development of the sporozoites and trophozoites in larvae is intracellular (Shortt and Swaminath 1927).

Ascogregarina mackiei **(Shortt and Swaminath 1927)** was described by Shortt and Swaminath (1927) from *Phlebotomus argentipes* Ann. and Brun. 1908 in India and later by Missiroli (1929; 1932) from *Ph. papatasi* (Scopoli 1786) in Italy. It is the only sand fly gregarine with intracellular development. In larvae, four gregarine stages can be found: ingested oocysts, sporozoites released from these oocysts, intracellular stages (all in the intestine) and adult gregarines in the intestine (often in the ectoperitrophic space) or the body cavity. The sporozoites (leaf-shaped, 4.8 × 1.8 μm) are located mostly in the posterior part of the midgut; the authors postulate that most sporozoites do not survive defecation, and therefore only a certain number of them is able to invade the host epithelial cells. The intracellular gregarines (23.4 μm) are initially round and later become triangular with no special organ of attachment. They concentrate mostly in the posterior intestine, and they are released from the epithelial cells into either the

intestinal lumen or the body cavity. The gamonts (round, oval or pear-shaped, 101.4 × 78 μm), with distinct nucleus (30 μm) and nucleolus (8 – 10 μm), are motile with well-marked longitudinal striations (Shortt and Swaminath 1927).

Fig. 4. The life cycle of *Ascogregarina chagasi*. Life cycle stages: (a) extracellular trophozoite attached to the larval gut epithelium; (b) gamonts; (c) syzygy; (d) young gametocyst; (e) gametocyst with oocysts; (f) oocysts. Location: (a) the 1st instar larval gut; (b) larval and pupal gut, pupal and adult body cavity; (c – e) larval gut, the body cavity of adults, gametocysts attached to the accessory glands of females; (f) larval gut and faeces, the body cavity of adults, lumen of the accessory glands of females, on the exochorion of eggs.



In pupae, only gamonts of *As. mackiei* occur, mostly in the posterior body cavity but never in the alimentary canal. The body reconstitution of the pupae probably causes passive relocation of the gregarines from the intestine into the body cavity; however, the authors point out that, in order to prove this, the processes during pupation need to be studied (Shortt and Swaminath 1927).

In adults, all the parasite life stages including oocysts were found. The large fully-grown gamonts are located always in the body cavity, more often in the posterior part. However, they are never within the digestive tract, and they tend to cluster around the reproductive organs. Sexual development of the gregarines occurs irrespective of a blood meal intake. Gametocysts are 66.3 – 152.1 μm in diameter, spherical or oval, and

they are mostly located in the posterior two thirds of the body cavity attached to the common oviduct. Oocysts are broad spindle-shaped (9.6 × 5.8 μm), with knob-like projection at each pole. The pressure produced by the growth of oocytes and the gametocysts causes disruption of the gametocyst wall at the site where they are attached to the oviduct, and the oocysts are injected into its lumen (Shortt and Swaminath 1927). Wu remarks (as unpublished observation in Ostrovska et al. 1990) that *As. mackiei* was also found to have oocysts in the accessory glands.

The prevalence of infected sand flies was roughly 25% or less in nature, while in the laboratory colonies, where the chance of infection is much higher, it was almost 100% (Shortt and Swaminath 1927).

Ascogregarina chagasi **(Adler and Mayrink 1961)**, originally named *Monocystis chagasi*, was described from *L. longipalpis* females in Brazil by Adler and Mayrink (1961), who gave several morphological and life cycle features that differentiate this parasite from *As. mackiei*. *Ascogregarina chagasi* is the most studied sand fly gregarine. In the 1st instar larvae, sporozoites and young trophozoites are attached to the larval epithelium through an osmiophilic contact zone; they are never located intracellularly (Warburg and Ostrovska 1991). The sporozoites are surrounded by a two-layered pellicle with subpellicular microtubules and possess an apical complex with conoid. The oval gamonts (60 – 90 μm), on the other hand, have a three-layered pellicle forming longitudinal epicytic folds (Warburg and Ostrovska 1991). They were found in the ectoperitrophic space of the intestine of the 3rd and 4th instar larvae by Warburg and Ostrovska (1991) and in the ectoperitrophic space of the 1st, 2nd and 3rd and in the intestinal lumen of the 4th instar larvae by Coelho and Falcao (1964). The latter authors also recorded one gamont in the Malpighian tubules and some sexual developmental stages in the intestinal lumen of the 4th instar larvae. The gregarine is able to complete its life cycle in the larvae, and oocysts in their faeces are a source of horizontal transmission.

In pupae, only gamonts occur (Coelho and Falcao 1964). Similarly to Shortt and Swaminath (1927), also Warburg and Ostrovska (1991) suggest that the relocation of the gregarines occurs during pupation. In males, Adler and Mayrink (1961) recorded only gamonts, while Coelho and Falcao (1964) found all the developmental stages of *As. chagasi* (gamonts, syzygies, gametocysts and oocysts) in the body cavity and a few gamonts also in the intestine.

In females, all the gregarine stages were recorded. Adler and Mayrink (1961) found syzygies, gametocysts and oocysts of *As. chagasi* 2 – 4 days post blood meal, while Warburg and Ostrovska (1991) found them already 72 hours post blood meal. The smallest gamonts are 30 μm in diameter (those over 60 μm have a proboscis-like projection), and adult round or oval gamonts ready to undergo syzygy measure 72 – 120 μm in diameter. Their nucleus (28 μm) has a distinctive nucleolus (8 – 10 μm) (Adler and Mayrink 1961). Gametocysts measure 72 – 120 μm (Adler and Mayrink 1961) or 95 – 120 μm (Warburg and Ostrovska 1991) in diameter. The size of the oocysts varies markedly in descriptions by different authors; Adler and Mayrink (1961) state their measurements as 11.4 × 5.8 μm, while Warburg and Ostrovska (1991) as 12.7 × 7.5 μm. The cytoplasm of the developing oocysts contains amylopectin granules, and all the life cycle stages apart from the gametocysts have a diffuse actin-like protein in the cytoplasm, suggesting the involvement of actin in movement (Warburg and Ostrovska 1991).

The gametocysts are attached to the accessory glands of females, but Adler and Mayrink (1961) found them also in the ovaries. Injection of the oocysts into the accessory glands is enhanced by plasmatocyte humoral encapsulation; encapsulated gametocysts and the internal pressure of the developing oocysts, as mentioned also by Adler and Mayrink (1961), results in rupture of the gametocysts at the site where they are attached to the accessory glands, releasing the oocysts into the gland lumen (Warburg and Ostrovska 1989). The majority of the oocysts in the accessory glands are uninucleate, and the nuclear divisions occur after 24 hours in a moist chamber (Adler and Mayrink 1961). The oocyst distribution among batches and individual eggs is irregular (Adler and Mayrink 1961). There are two modes of vertical transmission of this gregarine; the larvae ingest either oocysts released from the accessory glands on the exochorion of eggs or the ones in the body cavity of dead sand flies.

Ascogregarina saraviae **Ostrovska, Warburg and Montoya-Lerma 1990** was described from females of *Lutzomyia lichyi* (Floch and Abonnenc 1950) in Colombia. Two or three gametocysts were attached to the accessory glands of each infected female. Oocysts (12.4 × 5.8 μm) are located in the gametocysts, glands or on the egg surface. The differences in the size and shape of the oocysts (those of *As. saraviae* have thinner walls and narrower midsections than those of *As. chagasi*) give evidence that *As. saraviae* is a different, new species (Ostrovska et al. 1990).

Other gregarines from sand flies. A gregarine found in *Lutzomyia vexatrix occidentis* in California (Ayala 1971) differs from *As. chagasi* in several morphological and life cycle characteristics suggesting that it is a new species; the gamonts are pear-shaped (74 – 180 × 58 – 95 μm) or spherical (68 – 180 μm), with a light nucleus (24 – 26 μm) and nucleolus (8.5 μm), the gametocysts are spherical (110 – 190 μm), and the oocysts are spindle-shaped (10.5 × 6 μm). This was the only gregarine with sexual development (formation of syzygies, gametocysts and oocysts) in adults triggered by a blood meal intake.

In Brazil, gregarines identified as *As. chagasi* have been recorded from several sand fly species: *L. townsendi* (Scorza and Carnevali 1981), *Lutzomyia evandroi* (Brazil and Ryan 1984), *Lutzomyia sallesi* (Coelho a Falcao 1964), *Lutzomyia sordelli* (Oliveira et al. 1991 in Brazil et al. 2002) and *Lutzomyia cruzi* (Brazil et al. 2002). Oliveira et al. (1991) in Brazil et al. (2002) also found a gregarine in *Lutzomyia schreiberi*, which they consider *As. saraviae*.

A number of studies recorded unidentified gregarine species from *Lutzomyia shanonni* in Belize (Garnham and Lewis 1959), ten species of *Phlebotomus* sp. in Panama (McConnell and Correa 1964), *Lutzomyia cruciata* in Belize (Lewis 1965), *Lutzomyia flaviscutellata* in Brazil (Lewis et al. 1970), six species of *Lutzomyia* sp. in Brazil (Mayrink et al. 1979) and *Lutzomyia apache* in Wyoming (Reeves et al. 2008). Furthermore, Young and Lewis (1977) found gregarines in more that 20 sand fly species and Killick-Kendrick et al. (1976) found a neogregarine from the fat body of *Ph. ariasi*.

2.3.4 Host specificity of mosquito and sand fly gregarines

The host specificity studies of mosquito ascogregarines give rather contradictory results; some authors find these parasites fairly host specific, while others do not. *Ascogregarina lanyuensis* is not a host specific ascogregarine; it completed its life cycle in ten experimentally infected mosquito species, and in five of them, the prevalence was 100% (Jacques and Beier 1982). Also *As. geniculati* was able to develop in other mosquito species; after experimental infections, oocysts were found in *Ae. sierrensis*, *Ae. aegypti* and *Ae. triseriatus* (Munstermann and Levine 1983). *Ascogregarina barretti* developed in *Ae. geniculatus* (Rowton and Munstermann 1984) and *Ae. hendersoni*

(Copeland and Craig 1992), and Spencer and Olson (1982) were able to infect *Aedes epactius* with *As. culicis*.

Several authors showed that *As. taiwanensis* is not a host specific ascogregarine either. It infected 100% of *Ae. aegypti* and *Aedes taeniorhynchus* larvae, and oocysts were recovered from *Ae. taeniorhynchus* adults. On the other hand, three *Culex* species and one *Anopheles* species were not susceptible (Garcia et al. 1994). Munstermann and Wesson (1990) found *As. taiwanensis* in *Ae. epactius* and *Culex restuans* in Illinois, and after experimental infections, oocysts were recovered from *Ae. aegypti* and *Aedes atropalpus*, with the latter being the most susceptible of all experimentally infected mosquitoes (Munstermann and Wesson 1990). *Ascogregarina taiwanensis* was even able to develop in a sabethine mosquito *Wyeomyia smithii* (Diptera: Culicidae). Although the infection rates were low, gamonts persisted in the larval midgut for more than 37 days, and gametocysts were recovered from one female (Reeves and McCullough 2002).

Contrary to the above presented information, several authors showed that mosquito ascogregarines are host specific. During cross-infections of *Ae. sierrensis* with *As. culicis* and *Ae. aegypti* with *As. clarki*, neither of the two ascogregarine species developed fully (production of oocysts) in their non-natural hosts (Sanders and Poinar 1973). Oocysts of *As. armigerei*, *As. culicis*, *As. lanyuensis* and *As. taiwanensis* were used to infect *Ae. aegypti*, *Ae. albopictus*, *Ae. alcasidi* and *Ar. subalbatus*. Even though the trophozoites of all ascogregarines were recorded in all but one mosquito species (with lower infection rates in non-natural hosts), the oocysts were, apart from their natural hosts, recovered only from *Ae. alcasidi* for *As. taiwanensis* and *As. armigerei* (Lien and Levine 1980). This shows that *As. taiwanensis* is not, unlike the other gregarines from this study, host specific and supports other data about its low host specificity (see above). On the other hand, these results contradict Jacques and Beier (1982), who showed no host specificity of *As. lanyuensis*. Other discrepancies can be found for *As. geniculati*; this ascogregarine is not host specific according to Munstermann and Levine (1983); however, it could not infect *Aedes communis*, *Aedes cantans* and *Culex pipiens* (Kramar 1952).

Susceptibility of *Ae. aegypti* to *As. culicis* varies among geographical strains (Sulaiman 1992; Reyes-Villanueva et al. 2003); furthermore, one strain of *Ae. aegypti* from Trinidad was not susceptible to a Florida strain of *As. culicis* as no oocysts developed, while other Trinidad strains were susceptible (Beier et al. 1995).

As the host specificity of mosquito ascogregarines is unclear, methods that would differentiate between individual species were studied. Several morphological features (particularly the character of pigmentation and shape) distinguishing between gamonts of *As. culicis* and *As. taiwanensis* were found (Reyes-Villanueva et al. 2001). Oocysts of *As. armigerei*, *As. culicis*, *As. taiwanensis* and *Ascogregarina* sp. from *O. japonicus* were compared under scanning electron microscope, showing that they differ mainly in the length and structure of their surface (Roychoudhury et al. 2007a).

Ascogregarina barretti and *As. geniculati* were differentiated by isoenzyme electrophoresis (Rowton and Munstermann 1984); different migration rates were observed for isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogenase, and the authors find this method reliable for distinguishing between the two ascogregarines. A species-specific PCR method for *As. culicis* and *As. taiwanensis*, based on amplification of ribosomal ITS1 and ITS2 regions, was developed by Morales et al. (2005); the PCR products differed by at least 100bp. Furthermore, the authors found a diagnostic PCR method for the presence of ascogregarines in mosquitoes.

Unlike for widely studied mosquito ascogregarines, there is only one study evaluating sand fly gregarines proving their strict host specificity. Seven phlebotomine species were infected with oocysts of *As. chagasi* – *Ph. papatasi*, *Ph. argentipes*, *Ph. perniciosus*, *Lutzomyia serrana*, *Lutzomyia abonnenci*, *Lutzomyia columbiana* and gregarine-free *L. longipalpis*. In the Old World sand flies, trophozoites and no other gregarine life stages occurred only in the newly emerged adults of *Ph. papatasi*. Out of the New World *Lutzomyia* species, *L. columbiana* was the most susceptible non-natural host, and trophozoites were found in adults, as well as in *L. serrana* and *L. longipalpis*. *Ascogregarina chagasi* did not develop in *L. abonnenci*, and oocysts were found only in its natural host *L. longipalpis*. Furthermore, different strains of *L. longipalpis* varied in the susceptibility to *As. chagasi* (Wu and Tesh 1989). The authors argue that the gregarines from a number of New World sand flies identified as *As. chagasi* (see chapter 2.3.3) are new species.

2.3.5 Effects of gregarines on mosquitoes and sand flies

Similarly to host specificity, also studies evaluating effects of ascogregarines on their dipteran hosts give contradictory conclusions. One of the first records about ascogregarines being pathogenic brought Barrett (1968); *Ae. aegypti* larvae and pupae

infected with *As. culicis* were stunted, and the author noticed increased mortality. Sulaiman (1992) also observed increased larval mortality (proportional to the infection intensity) and shortened larval development; however, *As. culicis* did not affect larval development, size, mortality, pupal weight or adult emergence of *Ae. aegypti* in a study by McCray et al. (1970). *Ascogregarina barretti* reduces female pupal weight, prolongs development of males (Beier 1983) and increases the likelihood of pupal mortality (Siegel et al. 1992). Contrastingly, emergence success (Walker et al. 1987; Copeland and Craig 1992) or larval developmental time (Walker et al. 1987) are not affected. In addition, Beier (1983) and Copeland and Craig (1992) did not record any increase in the larval mortality of *As. barretti*-infected *Ae. triseriatus*.

In general, *As. barretti* is not very pathogenic to its adult host; it does not alter the size of males and females (Walker et al. 1987) or their survival (Beier 1983). However, the wing length of both sexes is reduced (Siegel et al. 1992). *Ascogregarina taiwanensis* has a little impact on adults of *Ae. albopictus* (Garcia et al. 1994), and *As. culicis*-infected *Ae. aegypti* do not have decreased survival or fecundity (McCray et al. 1970).

Parasitism by *Ascogregarina* sp. affects hosts to a larger degree when they are bred in nutrient-deficient conditions: the developmental time of mosquito females is prolonged, the mortality of larvae and blood-fed females is significantly increased, the size of females and males is significantly smaller, and the females produce fewer eggs (Walker et al. 1987; Comiskey et al. 1999a; 1999b). On the other hand, emergence rate, developmental time and the size of males are not affected (Walker et al. 1987). This sex-specific pattern of mosquito reaction to ascogregarine infection was supported by Tseng (2004).

The level of pathogenicity of mosquito ascogregarines is not influenced only by the sex or nutrients, but can be significantly greater when the gregarine is introduced to a non-natural host. For example, *As. taiwanensis* significantly increases the mortality of its non-natural host *Ae. taeniorhynchus*, while the mortality of *Ae. albopictus* is not affected (Garcia et al. 1994). *Ascogregarina barretti* decreases the larval survival, emergence success and female weight of non-natural *Ae. hendersoni*, while *Ae. triseriatus* is not influenced (Copeland and Craig 1992). Furthermore, not the natural host *Ae. aegypti*, but a non-natural host *Ae. epactius* is negatively affected by simultaneous *As. culicis* infection and methoprene, and the mortality rates are

significantly increased with increased methoprene concentrations (Spencer and Olson 1982).

The cause of ascogregarine pathogenicity to their dipteran hosts is probably the negative impact on the tissues where the parasites develop. Intestinal epithelial cells of mosquito larvae, the site of infection of intracellular sporozoites and trophozoites, can have enlarged nuclei (Kramar 1952) and can be destroyed by the gregarine parasitism (Kramar 1952; Sanders and Poinar 1973). The Malpighian tubules of adults, the site of infection of extracellular trophozoites and subsequent developmental stages, are dilated (Wenyon 1911), and their cells are distorted and damaged (Barrett 1968; McCray et al. 1970; Sanders and Poinar 1973). The extent of the damage is proportional to the infection rate, and already eight to 25 gametocysts may destroy one third of a single Malpighian tubule (Barrett 1968).

Ascogregarines may a play role in the ability of mosquitoes to invade new areas. When *Ae. aegypti* and *Ae. albopictus* are coexisting in the same habitat, the replacement of the former by the latter occurs (Dos Passos and Tadei 2008). Two complimentary facts could explain this phenomenon. (1) According to Aliabadi and Juliano (2002), the survivorship of *Ae. triseriatus* larvae is reduced when they are being bred with invasive gregarine-non-infected *Ae. albopictus* larvae, and it is not affected when breeding with infected *Ae. triseriatus*. (2) Blackmore et al. (1995) showed that when *Ae. albopictus* is introduced to a new region, parasitism by *As. taiwanensis* is low at the beginning and becomes higher after three years. Therefore, this phase of lower infection rate may give competitive advantage to *Ae. albopictus* over *Ae. aegypti* enabling its expansion.

The ability of ascogregarine-infected mosquitoes to transmit parasites and viruses has been studied. Mourya et al. (2003) showed that Chikungunya virus (Togaviridae) could be vertically transmitted to *Ae. aegypti* through *As. culicis* oocysts. Studies evaluating the effects of *As. taiwanensis* on the development of *Dirofilaria immitis* (Nematoda: Onchocercidae) have come to contradictory conclusions. Comiskey et al. (1999b) found that in high nutrient conditions, *D. immitis* infective rate in *Ae. albopictus* females co-infected with *As. taiwanensis* is significantly higher than in females infected only with *D. immitis*, suggesting that *As. taiwanensis* increases the vector competence of *Ae. albopictus* for filariae. Contrastingly, Beier (1983) did not observe any significant differences in the number of infective *D. immitis* larvae between gregarine-infected and non-infected mosquitoes.

Contrastingly to the good number of studies evaluating the effects of mosquito ascogregarines to their hosts, there is only a single work dealing with this topic in sand flies. *Ascogregarina chagasi* significantly decreased the survival of *L. longipalpis* females; the difference was evident from the day eight of the experiment. On the 25th day, the survivorship for the control females was 77.1% and for the gregarine-infected females only 49.5%. *Ascogregarina chagasi* did not significantly affect the fecundity of its host (Wu and Tesh 1989).

3 PUBLICATIONS

Votypka J., **Lantova L.**, Ghosh K., Braig H., Volf P. 2009. **Molecular characterization of gregarines from sand flies (Diptera: Psychodidae) and description of *Psychodiella* n. g. (Apicomplexa: Gregarinida)**. The Journal of Eukaryotic Microbiology 56: 583-588.

Lantova L., Ghosh K., Svobodova M., Braig H. R., Rowton E., Weina P., Volf P., Votypka J. 2010. **The life cycle and host specificity of *Psychodiella sergenti* n. sp. and *Ps. tobbi* n. sp. (Protozoa: Apicomplexa) in sand flies *Phlebotomus sergenti* and *Ph. tobbi* (Diptera: Psychodidae)**. Journal of Invertebrate Pathology 105: 182-189.

Lantova L., Volf P. 2011. **The development of *Psychodiella sergenti* (Apicomplexa: Eugregarinorida) in *Phlebotomus sergenti* (Diptera: Psychodidae)**. Submitted in Parasitology (20th May 2011).

Lantova L., Svobodova M., Volf P. 2011. **Effects of *Psychodiella sergenti* (Apicomplexa, Eugregarinorida) on its natural host *Phlebotomus sergenti* (Diptera, Psychodidae)**. Accepted in Journal of Medical Entomology (26th June 2011).

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Molecular Characterization of Gregarines from Sand Flies (Diptera: Psychodidae) and Description of *Psychodiella* n. g. (Apicomplexa: Gregarinida)

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ABSTRACT. Sand fly and mosquito gregarines have been lumped for a long time in the single genus *Ascogregarina* and on the basis of their morphological characters and the lack of merogony been placed into the eugregarine family Lecudinidae. Phylogenetic analyses performed in this study clearly demonstrated paraphyly of the current genus *Ascogregarina* and revealed disparate phylogenetic positions of gregarines parasitizing mosquitoes and gregarines retrieved from sand flies. Therefore, we reclassified the genus *Ascogregarina* and created a new genus *Psychodiella* to accommodate gregarines from sand flies. The genus *Psychodiella* is distinguished from all other related gregarine genera by the characteristic localization of oocysts in accessory glands of female hosts, distinctive nucleotide sequences of the small subunit rDNA, and host specificity to flies belonging to the subfamily Phlebotominae. The genus comprises three described species: the type species for the new genus—*Psychodiella chagasi* (Adler and Mayrink 1961) n. comb., *Psychodiella mackiei* (Shortt and Swaminath 1927) n. comb., and *Psychodiella saraviae* (Ostrovska, Warburg, and Montoya-Lerma 1990) n. comb. Its creation is additionally supported by sequencing data from other gregarine species originating from the sand fly *Phlebotomus sergenti*. In the evolutionary context, both genera of gregarines from mosquitoes (*Ascogregarina*) and sand flies (*Psychodiella*) have a close relationship to neogregarines; the genera represent clades distinct from the other previously sequenced gregarines.

Key Words. Accessory glands, *Ascogregarina*, *Lutzomyia*, neogregarines, parasite, *Phlebotomus*, SSU rDNA phylogeny.

GREGARINES represent an extremely large, diverse, and highly abundant group of early branching apicomplexans that are widely distributed in marine as well as in terrestrial invertebrates. They parasitize annelids, mollusks, nemerteans, phoronids, echinoderms, siphunculids, crustaceans, hemichordates, appendicularians, and insects. Traditionally, three gregarine groups are recognized according to differences in habitat, host range, and morphological features of the trophozoites: archigregarines, eugregarines, and neogregarines (Vivier and Desportes 1990).

The genus *Ascogregarina* Ward, Levine, and Craig 1982 (syn. *Monocystis* von Stein 1848; *Lankesteria* Mingazzini 1891, and *Ascocystis* Grassé 1953) belongs to the order Eugregarinida Léger, 1899 (class Gregarinida Duffour 1828; phylum Apicomplexa Levine 1970). Out of 16 named species of the genus (Clopton 2000), three species parasitize sand flies and nine species parasitize mosquitoes (Levine 1977, 1985, 1988; Ostrovska et al. 1990). The total number of species is, however, questionable, because Clopton (2000) did not respect the definition of Ormières (1965) who restricted the genus only to parasites of Diptera. The terminology of mosquito and sand fly gregarines is complicated and the history of final designations is quite long. The mosquito gregarine and type species of the genus is *Ascogregarina culicis* (Ross 1898), originally named as *Gregarina culicis*. Ross (1895) described this species from the yellow fever mosquito *Aedes aegypti* (Linnaeus) as *Gregarina culicidis*, but this is considered as a *lapsus calami*. Wenyon (1911) reclassified the species as *Lankesteria culicis* and Grassé (1953) proposed the name *Ascocystis* for gregarines of insects that had formerly been assigned to the genus *Lankesteria*, and reclassified the species as *Ascocystis culicis*. The genus *Ascocystis* was later reviewed by Ormières (1965), who accepted *Ascocystis* Grassé, 1953 for parasites of Diptera and restricted *Lankesteria* to parasites of ascidians. However, Ward et al. (1982) established the name *Ascogregarina* instead of *Ascocystis* because the name was pre-occupied by a fossil crinoid echinoderm.

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Phlebotomine sand flies (Diptera: Psychodidae) of the genera *Phlebotomus* Rondani and Berté and *Lutzomyia* França are important vectors of human diseases, namely leishmaniasis, bartonellosis, and sand fly fever virus infections (Adler and Theodor 1957). Their larvae develop in soil rich in humus and microorganisms. Gregarines have been reported from more than 20 sand fly species (Ayala 1971; Levine 1977; Lisova 1962; Ostrovska et al. 1990; Tuzet and Rioux 1966; Warburg and Ostrovska 1991; Wu and Tesh 1989; Young and Lewis 1977), but only a few of them were denominated. The first sand fly gregarine was described as *Monocystis mackiei* Shortt and Swaminath 1927 from *Phlebotomus argentipes* (Annandale and Brunetti) in India. A few years later, presumably the same gregarine species was found in *Phlebotomus papatasi* (Scopoli) in Italy and renamed by Missiroli (1929, 1932) as *Lankesteria phlebotomi mackiei*. Ormières (1965) and 1 year later, Tuzet and Rioux (1966) reclassified the species as *Ascocystis mackiei*. However, the current name should be *Ascogregarina mackiei*, according to Ward et al. (1982).

The well-known sand fly gregarine described as *Monocystis chagasi* Adler & Mayrink, 1961 was found first in the hemocoel and accessory gland of *Lutzomyia longipalpis* (Lutz and Neiva) in Brazil and later in four other Neotropical sand fly species (Brazil and Ryan 1984; Coelho and Falcao 1964; Lewis, Lainson, and Shaw 1970; Scorza and Carnevali 1981). Tuzet and Rioux (1966) reclassified the species as *Ascocystis chagasi* and according to Ward et al. (1982) the current name should be *Ascogregarina chagasi*. The third ascogregarine species from sand flies was described by Ostrovska et al. (1990) as *Ascogregarina saraviae* Ostrovska, Warburg, & Montoya-Lerma, 1990 from *Lutzomyia lichyi* (Floch and Abbonenct).

Ascogregarina chagasi and other members of the genus were placed among the eugregarines (Apicomplexa: Conoidasida: Gregarinida: Eugregarinida: Aseptatorina: Lecudinidae) exclusively on the basis of morphological features and part of their developmental biology in the host. Their phylogenetic position has not been analyzed yet, despite Roychoudhury et al. (2007) having published sequences of four mosquito ascogregarines, including the type species *A. culicis*. DNA sequences generated by the present work enable phylogenetic analysis of the sand fly gregarines and provide more depth to our understanding of relatedness among gregarine groups.

MATERIALS AND METHODS

Sand flies. Sand fly colonies were maintained independently in the Czech Republic and in the United States. The colonies of *L. longipalpis* originated from Jacobina village, Bahia, Brazil (11°11′S, 40°32′W). The colonies of *Phlebotomus sergenti* originated from SanliUrfa city, Turkey (37°11′N, 38°48′E). Standard maintenance of colonies was described by Benkova and Volf (2007) for the Czech colonies and by Modi and Tesh (1983) for the colonies that were maintained at the Walter Reed Army Institute of Research, Silver Spring, MD.

Gregarine isolation and identification. All the molecular work was performed independently and in parallel in the Czech Republic and the United States. Two to 5-day-old adult flies of both sexes from colonies of *L. longipalpis* and *P. sergenti* were washed by 1.5% (v/v) Triton X-100 to remove any microorganisms and body hairs from the surface and by distilled water and phosphate-buffered saline (PBS). Approximately 50 gametocysts of each gregarine species were dissected under the stereo microscope (SZH-ILLD, Olympus Optical Co. Ltd., Tokyo, Japan) in NET-50 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and 5 mM EDTA) and stored in −70 °C for DNA extraction. To confirm that gregarines found in both our colonies of *L. longipalpis* are of the species *Psychodiella* (formerly *Ascogregarina*) *chagasi*, adult sand flies of both sexes were dissected in PBS under the stereo microscope connected to a digital camera (DP-70, Olympus) for photo documentation of native preparations. Morphological characters of gregarine gamonts, gametocysts, and oocysts were evaluated using light microscope (BX-50, Olympus Optical Co. Ltd.).

DNA extraction, polymerase chain reaction (PCR) amplification, and cloning. Extraction of the total DNA from the pool of gregarine parasites was performed using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. The small subunit (SSU) rRNA genes were amplified as a single fragment using universal eukaryotic primers (Medlin et al. 1988). The PCR reactions were performed in 25 µl total volumes of reaction mix (Combi PPP Master Mix, Top-Bio, Prague, Czech Republic; Promega, Fitchburg, WI) using the following conditions: initial denaturation at 95 °C for 5 min followed by 30 amplification cycles (95 °C for 60 s, 55 °C for 90 s, and 72 °C for 90 s) and 72 °C for 7 min.

The PCR products corresponding to the expected size were gel isolated (Gel extraction kit, Qiagen, Valencia) and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD) using the manufacturer's protocol. Three clones from each gregarine species were sequenced with Terminator Ready Reaction Mix (Promega, Fitchburg, WI) using the vector primers and four internal primers oriented in both directions. The sequencing reaction was carried out on an automated DNA sequencer (310 Genetic Analyzer; ABI Prism, Foster City, CA) using the BigDye 3.1 kit (Applied Biosystems, Foster City, CA). Gregarine sequences were deposited in GenBank under the following accession numbers: FJ865354 (*P. chagasi* n. comb.) and FJ865355 (*Psychodiella* sp. from *P. sergenti*).

Phylogenetic analysis. The data set containing 38 nominal SSU rRNA gene sequences was used to establish the phylogenetic position of both newly sequenced sand fly gregarines (Table 1). The DNA sequences were compared with those in the GenBank database using the BLAST algorithm. All sequences of the SSU rRNA genes of gregarines available in public databases except incomplete sequences were used in our analysis. Appropriate sequences were aligned using the program CLUSTAL X 1.81. Alignment was manually edited using the program BioEdit 5.0.9.; gaps, as well as ambiguously aligned regions, were omitted from further analysis. The alignment is available from the corresponding author upon request. Phylogenetic analysis was performed using maximum parsimony (MP; PAUP *4.0b10; Swofford 2002) by 10 replicates of heuristic search, maximum likelihood (ML; PhyML; Guindon and Gascuel 2003), and Bayesian method (MrBayes; Huelsenbeck and Ronquist 2001). The MP bootstrap analyses were performed with 1,000 replicates. The ML trees were constructed using the GTR model for nucleotide substitutions with γ-distribution in 8+1 categories. The models of

Table 1. Origin of SSU rRNA gene sequences analyzed.

Species	Order[a]	Accession number	Host species
Selenidium serpulae	A	DQ683562	*Serpula vermicularis*
Selenidium terebellae	A	AY196709	*Thelepus* sp.
Selenidium vivax	A	AY196708	*Phascolosoma agassizii*
Ascogregarina armigerei	E	DQ462459	*Armigeres subalbatus*
Ascogregarina culicis (Thailand)	E	DQ462456	*Aedes aegypti*
Ascogregarina culicis (Viet Nam)	E	DQ462457	*Aedes aegypti*
Ascogregarina taiwanensis (Japan)	E	DQ462454	*Aedes albopictus*
Ascogregarina taiwanensis (India)	E	DQ462455	*Aedes albopictus*
Ascogregarina sp.	E	DQ462458	*Ochlerotatus japonicus*
Gregarina caledia[b]	E	L31799	
Gregarina chortiocetes[b]	E	L31841	
Gregarina niphandrodes	E	AF129882	*Tenebrio molitor*
Gregarina polymorpha	E	AF457129	*Tenebrio molitor*
Lankesteria abbotti	E	DQ093796	*Cnemidocarpa* sp.
Lankesteria chelyosomae	E	EU670240	*Chelyosoma columbianum*
Lankesteria cystodytae	E	EU670241	*Cystodytes lobatus*
Lecudina tuzetae	E	AF457128	*Nereis vexillosa*
Lecudina polymorpha type 1	E	AY196706	*Lumbrineris* sp.
Lecudina polymorpha type 2	E	AY196707	*Lumbrineris* sp.
Leidyana migrator	E	AF457130	*Gromphadorhina portentosa*
Lithocystis sp.	E	DQ093795	*Brisaster latifrons*
Monocystis agilis	E	AF457127	*Lumbricus terrestris*
Pseudomonocystis lepidiota[b]	E	L31843	
Psychodiella chagasi	**E**	**FJ865354**	***Lutzomyia longipalpis***
***Psychodiella* sp.**	**E**	**FJ865355**	***Phlebotomus sergenti***
Pterospora floridiensis	E	DQ093794	*Axiothella mucosa*
Pterospora schizosoma	E	DQ093793	*Axiothella rubrocincta*
Mattesia geminata	N	AY334568	*Solenopsis geminata*
Mattesia sp.	N	AY334569	*Solenopsis invicta*
Ophriocystis elektroscirrha	N	AF129883	*Danaus plexippus*
Syncystis mirabilis	N	DQ176427	*Nepa cinerea*
Cryptosporidium baileyi		L19068	birds
Cryptosporidium muris		L19069	rodents
Cryptosporidium parvum		AF112569	primates
Apicomplexan pathogen		AY490099	*Acarus siro*
Environmental sample		AY179988	water and sediments
Environmental sample		AY821921	water and sediments
Environmental sample		EF100358	water and sediments

[a]A, Archigregarinida; E, Eugregarinida; N, Neogregarinida.

[b]These three taxa have been included as names of environmental samples, they do not represent named species. The authors of those sequences did not provide any hosts.

New sequences reported in this work are in bold.

nucleotide substitution for maximum likelihood was chosen by hierarchical nested likelihood ratio tests implemented in Modeltest 3.06 and bootstrap analysis was computed in 1,000 replicates using the same model with Γ-distribution in four categories and all parameters (the proportion of invariant sites, γ shape parameter, TS/TV ratio for purines and pyrimidines) estimated from the data set. The Bayesian analysis was performed using MrBayes 3.1.2. Base frequencies, rates for six different types of substitution, proportion of invariant sites, and shape parameter of the γ correction for the rate heterogeneity with four discrete categories were allowed to vary. The covarion model was used to allow rate heterogeneity along the tree. The number of generations of Markov chain Monte Carlo was 5×10^6 and the trees were sampled every 100th generation. The first 12,500 trees were discarded as burn-in.

RESULTS

Features of SSU rDNA gene sequences. The identical SSU rRNA gene sequences of *P. chagasi* n. comb. from the colony of *L. longipalpis* (Jacobina village, Brazil) and the identical sequences of *Psychodiella* sp. from the colony of *P. sergenti* (SanliUrfa City, Turkey), were independently obtained from the USA and from the Czech Republic, respectively. The full length of the SSU rRNA gene sequences deposited in GenBank is 1,749 base pairs for *P. chagasi* (under the accession number FJ865354) and 1,752 base pairs for *Psychodiella* sp. from *P. sergenti*, respectively.

Phylogenetic analysis. Comparison of available gregarine sequences in GenBank revealed that the sequences of *P. chagasi* n. comb. and the most closely related *Psychodiella* sp. from *P. sergenti* form well supported clade (Fig. 1). Sequence divergence between the New World *P. chagasi* from *L. longipalpis* and the Old World *Psychodiella* sp. from *P. sergenti* is about 2% (35 changes). In our analysis, all tree topologies inferred using MP, ML, and Bayesian method were basically congruent. Cryptosporids were used as an outgroup. Within gregarine lineage, *Psychodiella* spp. and *Ascogregarina* spp. formed monophyletic clades supported by high bootstrap values (MP, 100%; ML, 100%; BA, 1.00). Phylogenetic analyses clearly demonstrate disparate position of gregarines parasitizing mosquitoes (genus *Ascogregarina*) and gregarines from sand flies (genus *Psychodiella*).

Morphological features of *Psychodiella chagasi* parasitizing *Lutzomyia longipalpis*. All life cycle stages of the gregarine from *L. longipalpis* were clearly identified as or conformed to the species *P.* (formerly *Ascogregarina*) *chagasi* originally described by Adler and Mayrink (1961). The life cycle of the gregarine in adult sand flies is identical to this of *P. chagasi* (Adler and Mayrink 1961; Warburg and Ostrovska 1991); syzigies, gametocysts, and oocysts were found in the body cavity of both female and male adults while in females gametocysts were attached to accessory glands (Fig. 2). Ocysts are released from gametocysts into the lumen of the glands and when laying eggs, the content of accessory glands including oocysts are excreted on the eggshells providing transovarial transmission.

DISCUSSION

Molecular phylogeny of *Ascogregarina* and *Psychodiella* n. g. as inferred from SSU rDNA. Sand fly and mosquito gregarines have been considered for a long time as a single genus *Ascogregarina*. Up to now, nine species were described from mosquitoes and three from sand flies. It should be mentioned that all available gregarine sequences of the fly-infecting species are from nematoceran flies, a relatively small group within the Diptera. Even though sand fly and mosquito gregarines have been considered as eugregarines for a long time, phylogenetic analyses revealed that the SSU sequences of both genera (*Ascogregarina* and *Psychodiella*) do not show similarity to the Eugregarinida but to the Neogregarinida. Monophyly of both genera in our trees is supported by 100% bootstrap by all methods used, and the genera seem to represent two separate and dissimilar clusters within the gregarines.

The phylogenetic analyses of all available sequences of gregarines correspond with the findings of other authors (Leander 2007, 2008; Leander, Clopton, and Keeling 2003a; Leander, Harper, and Keeling 2003b; Leander et al. 2006; Rueckert and Leander 2008, 2009). The genera *Selenidium* and *Lecudina* form paraphyletic groups and monocystids (e.g. *Monocystis* that infect terrestrial annelids), traditionally considered to be aseptate eugregarines, tend to be included in the group of neogregarines (e.g. *Syncystis* and *Mattesia*). The monophyly and composition of the order Eugregarinida are uncertain, and this is especially because the SSU rDNA sequences of eugregarines tend to be highly divergent, forming long branches in our molecular phylogenetic analyses.

Gregarines are important from an evolutionary perspective because of their suspected early diverging position within the Apicomplexa. Their molecular phylogenetic data have added additional complexity (and uncertainty) to the deepest relationships among apicomplexans. An internal topology of the apicomplexans is just beginning to emerge from comparisons of morphological characteristics and gene sequences (e.g. Beck et al. 2008; Ellis, Morrison, and Jeffries 1998; Kuo, Wares, and Kissinger 2008; Šlapeta et al. 2003). However, most of this work has focused on representatives from three of the four major groups: coccidians, haemosporidians, and piroplasms. Most studies of the phylogenetic relationships of gregarines are based on a relatively restricted data set of SSU rDNA sequences (Carreno, Martin, and Barta 1999; Leander 2007, 2008; Leander et al. 2003a,b, 2006; Roychoudhury et al. 2007; Rueckert and Leander 2008; Valles and Pereira 2003). With the advancement of DNA technology as more genes or whole genomes are sequenced and more data become available, there will be a need in the future for revisiting the systematics of gregarines, a neglected but extremely numerous branch of apicomplexan parasites.

As determined from the SSU rRNA gene sequence-based analyses, the genus *Psychodiella* n. g. is a member of the class Gregarinida Dufour, 1828. However, it cannot be associated with any valid genus. The morphological appearance, overall shape and cell size of species belonging to this genus resembles that of the genus *Ascogregarina*, although in adult sand flies gregarines are not localized in Malpighian tubules like gregarines from mosquitoes. Moreover, the genus *Psychodiella* is distinguished from all other related gregarine genera in having characteristic localization of oocysts in the accessory glands of the female host, its distinctive nucleotide sequences of SSU rDNA (FJ865354 and FJ865355), and its host specificity to phlebotomine sand flies. The phylogenetic analyses indicate that *Psychodiella* and *Ascogregarina* evolved independently of each other.

In conclusion, sequence data do not justify the inclusion of sand fly gregarines in the genus *Ascogregarina* and therefore we propose in accordance with the rules of the zoological nomenclature ICZN to separate sand fly gregarines into a newly erected genus *Psychodiella* n. g. In an evolutionary framework, both genera of gregarines from mosquitoes (*Ascogregarina*) and sand flies (*Psychodiella*) have close relationship to neogregarines, but represent a distinct clade from other previously sequenced gregarines. Studies on gregarines isolated from other sand fly species are underway (Lantová et al., unpubl. data) in order to examine if they are morphologically and genetically related to *P. chagasi*.

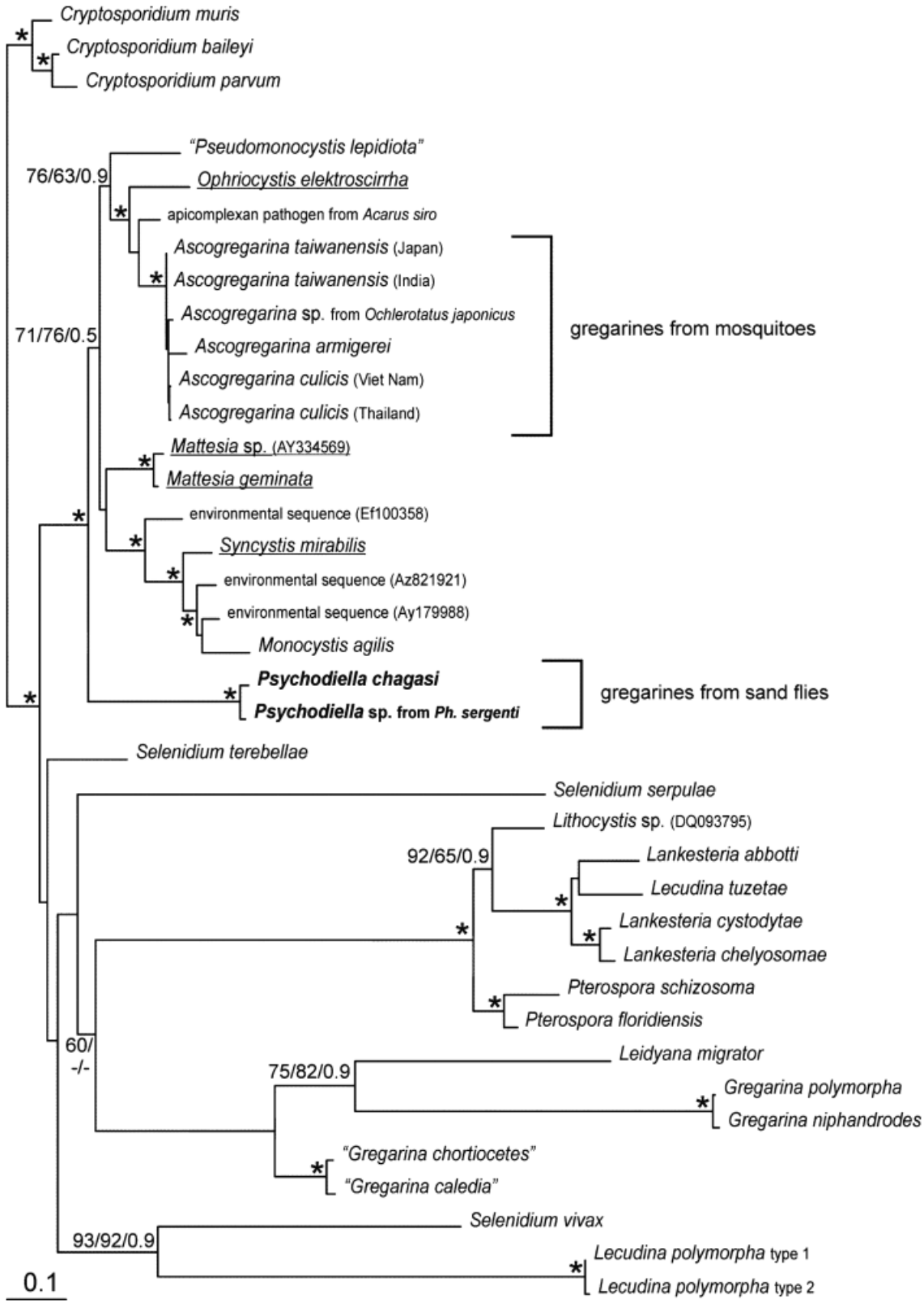

Fig. **1.** Maximum likelihood phylogenetic tree as inferred from small subunit rRNA gene sequences. The figure shows the topology for 38 taxa obtained by maximum likelihood using the GTR model for nucleotide substitutions with Γ-distribution in 8+1 categories as implemented in PhyML. Bootstrap values from maximum likelihood (100 replicates), maximum parsimony (1,000 replicates), and Bayesian posterior probabilities (number of generations was 5×10^6) are shown above branches, respectively. Asterisks (*) at the nodes denote Bayesian posterior probabilities and bootstrap percentages of 95% or higher. Dashes (-) indicate bootstrap support below 50 or posterior probability below 0.5 or different topology. The sequences of the species derived from this study are marked in bold. Gregarine species of the order Neogregarinida are underlined. Bars represent 0.1 substitutions per site. *Cryptosporidium* spp. served as an outgroup.

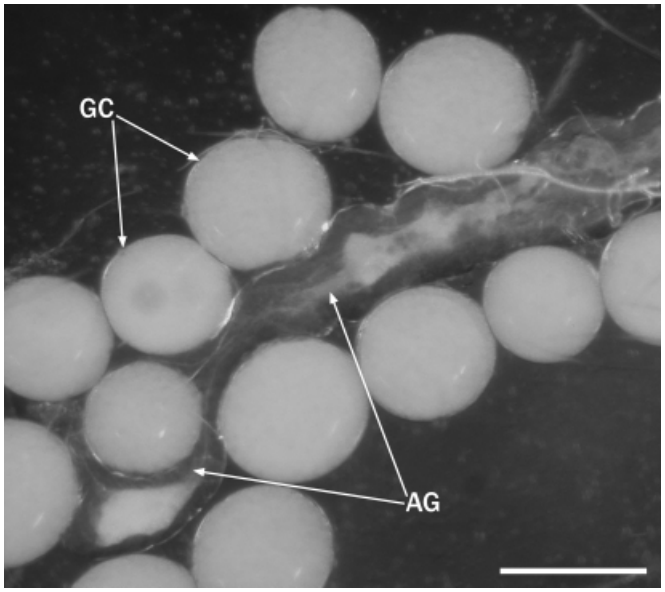

Fig. **2.** Gametocysts (GC) of *Psychodiella chagasi* n. comb. attached to accessory glands (AG) of an adult female of the phlebotomine sand fly *Lutzomyia longipalpis*. Native preparation. Scale bar = 100 μm.

TAXONOMIC SUMMARY

Phylum Apicomplexa
Order Eugregarinida
Suborder Aseptatorina

***Psychodiella* n. g. Votýpka, Lantová, and Volf**

Description. Monoxenous parasitic gregarine in Diptera. Gamonts oval, circular, or pear-shaped, aseptate, mucron not always apparent; gametocysts spherical or broad oval, in adults in the body cavity, in females usually attached to accessory glands; oocysts ellipsoidal or spindle shaped, often with a plug at each end, injected into accessory glands of female host.

Type species. *Monocystis chagasi* Adler & Mayrink 1961

Etymology. *Psychodiella*. The genus name has been derived from the name of the host family Psychodidae, the name is of feminine gender.

Remarks. The genus *Psychodiella* encompasses the following three species: *Psychodiella mackiei* (Shortt and Swaminath 1927) n. comb., *P. chagasi* (Adler and Mayrink 1961) n. comb., and *Psychodiella saraviae* (Ostrovska, Warburg, & Montoya-Lerma, 1990) n. comb.

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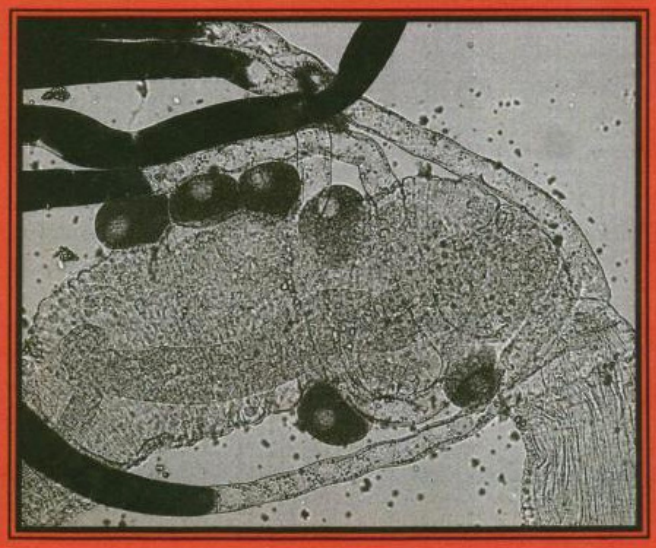

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SSU rDNA phylogeny

ABSTRACT

Two new gregarines in the recently erected genus *Psychodiella* (formerly *Ascogregarina*), *Psychodiella sergenti* n. sp. and *Psychodiella tobbi* n. sp., are described based on morphology and life cycle observations conducted on larvae and adults of their natural hosts, the sand flies *Phlebotomus sergenti* and *Phlebotomus tobbi*, respectively. The phylogenetic analyses inferred from small subunit ribosomal DNA (SSU rDNA) sequences indicate the monophyly of newly described species with *Psychodiella chagasi*. *Ps. sergenti* n. sp. and *Ps. tobbi* n. sp. significantly differ from each other in the life cycle and in the size of life stages. The sexual development of *Ps. sergenti* n. sp. (syzygy, formation of gametocysts and oocysts) takes place exclusively in blood-fed *Ph. sergenti* females, while the sexual development of *Ps. tobbi* n. sp. takes place also in males and unfed females of *Ph. tobbi*. The susceptibility of *Phlebotomus perniciosus*, *Phlebotomus papatasi*, *Ph. sergenti*, *Ph. tobbi*, and *Phlebotomus arabicus* to both gregarines was examined by exposing 1st instar larvae to parasite oocysts. High host specificity was observed, as both gregarines were able to fully develop and complete regularly the life cycle only in their natural hosts. Both gregarines are considered as serious pathogens in laboratory-reared colonies of Old World sand flies.

1. Introduction

The gregarine parasites have monoxenous life cycles and inhabit the intestines and other organs of invertebrates, particularly insects (Perkins et al., 2000). They are widespread in numerous hosts, but are currently not given the attention they deserve. Therefore, most gregarine species remain unknown and undescribed. The systematic status of gregarines has not been resolved and would greatly benefit from the addition of DNA sequences as molecular characters (Leander, 2008). Molecular phylogenetic data demonstrate that gregarines are more divergent than previously assumed and new lineages have emerged from older taxa (Leander, 2008; Votýpka et al., 2009).

Nematoceran Diptera are usually considered as rare hosts of gregarines and only 12 named species of two relatively morphologically uniform genera inhabit mosquitoes (*Ascogregarina*) and sand flies (*Psychodiella*) (Warburg and Ostrovska, 1991; Chen, 1999; Perkins et al., 2000; Votýpka et al., 2009). The genus *Psychodiella* encompasses three named species of the aseptate gregarines (order Eugregarinorida according to Perkins et al. (2000)) that parasitize sand flies. Its type species, *Ps. chagasi*, was described by Adler and Mayrink (1961) as *Monocystis chagasi* in the hemocoel and accessory glands of the New World sand fly *Lutzomyia longipalpis* in Brazil. All life stages, including gametocysts and oocysts, occur in larvae and both adult sexes. Larvae become infected by feeding on oocysts either attached to the chorion of eggs or released into larval habitats following the death and decay of infected adults. Sporozoites are released from the oocysts after ingestion by sand fly larvae, attach to the gut epithelial cells, and develop into trophozoites (Adler and Mayrink, 1961; Coelho and Falcao, 1964; Wu and Tesh, 1989). Natural infections by this gregarine species have also been recorded in four other Neotropical sand fly species: *Lutzomyia sallesi*, *Lutzomyia flaviscutellata*, *Lutzomyia townsendi*, and *Lutzomyia evandroi* (see Wu and Tesh, 1989; Ostrovska et al., 1990). The second Neotropical species, *Psychodiella saraviae*, was described by Ostrovska et al. (1990) as *Ascogregarina saraviae* from blood-fed females of *Lutzomyia lichyi* with gametocysts attached to accessory glands and oocysts in the lumen.

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The only Old World species, *Psychodiella mackiei*, was described as *Monocystis mackiei* by Shortt and Swaminath (1927) from *Phlebotomus argentipes* in India and 2 years later was isolated from *Ph. papatasi* in Italy (Missiroli, 1929, 1932).

Contradictory results have come from studies focused on host specificity of gregarines in nematoceran hosts. Levine (1977) suggested a broad host range of *Ps. chagasi* among the New World sand fly species, while Wu and Tesh (1989) demonstrated strict host specificity of this species. Low host specificity was suspected for the sand fly gregarine *Ps. mackiei*, as it was described from Indian *Ph. argentipes* (Shortt and Swaminath, 1927) and Italian *Ph. papatasi* (Missiroli, 1929). Garcia et al. (1994) have shown that *Ascogregarina* species are not host-specific parasites in mosquitoes, as oocysts from *Aedes albopictus* were infectious to both *Aedes aegypti* and *Ochlerotatus taeniorhynchus* larvae. Similar results were obtained by Jacques and Beier (1982) infecting various *Aedes* species with *Ascogregarina lanyuensis* (Lien and Levine, 1980). On the other hand, a high degree of host specificity of these mosquito gregarines was demonstrated in cross-infection studies by Lien and Levine (1980) and Reyes-Villanueva et al. (2003).

Although most gregarines are often considered as non-pathogenic to their natural hosts (Henry, 1981), their impact on infected insects is not always clear (Clopton, 1995), notably when infection levels within a population are high as sometimes happens in reared colonies (e.g., Klingenberg et al., 1997). In these situations, the overall number of sand flies produced drops (personal observations, Rowton and Lawyer). In mosquitoes, however, some gregarine species are clearly pathogenic (Sulaiman, 1992; Comiskey et al., 1999) and could potentially serve as disease agents for biological control (Perkins et al., 2000). In sand flies, negative impact on adult longevity was described for *Ps. chagasi* (Wu and Tesh, 1989). This species is known as common pathogen in laboratory-reared colonies of *L. longipalpis* (Dougherty and Ward, 1991).

It is notable that descriptions of sand fly or mosquito gregarines lack a molecular phylogenetic approach and all descriptions of *Psychodiella* species from sand flies lack cross-infection studies. The present work describes not only the life cycle and morphology of the two newly described gregarine species from *Ph. sergenti* and *Ph. tobbi*, but also reveals their phylogenetic position and their ability to infect other sand fly species in cross-infections.

2. Materials and methods

2.1. Parasites

Gregarines were obtained from laboratory-reared sand fly colonies maintained using the methods of Benkova and Volf (2007). However, these colonies were established from naturally infected sand fly females originated from different places. Gregarines of the species *Ps. chagasi* were obtained from *L. longipalpis* collected in Jacobina, Bahia, Brazil, *Ps. sergenti* n. sp. from *Ph. sergenti* collected in Sanli Urfa, South-East Anatolia, Turkey, and *Ps. tobbi* n. sp. from *Ph. tobbi* collected in Tepecikoren, near Adana city, South Anatolia, Turkey. Prevalence of gregarines in wild-caught females was determined based on dissection of sand flies in the frame of leishmania-detection studies.

2.2. Light microscopy

Up to 30 specimens each of: 4th instar larvae, 1–10 day-old males, 1–7 day-old unfed females (no previous blood meal), and females 3–7 days after a blood meal were dissected in phosphate-buffered saline (PBS) under a stereomicroscope (SZH-ILLD, Olympus). The shape and the size of gamonts, gametocysts, and oocysts of gregarines from adult sand flies were measured under an optical microscope (SBX-50, Olympus). Light micrographs were produced with a DP-70 digital camera (Olympus) and measurements were processed with QuickPHOTO MICRO 2.2 software (Olympus). The statistical evaluation and difference of measurements among gregarine stages from the three sand fly species studied was determined using Statistica 6.0 (StatSoft).

2.3. Experimental infections

Development of both newly described gregarine species was studied in five laboratory-reared sand fly species (see Table 1). Oocysts were obtained by homogenization of 25–30 adults in 500 μl of PBS. In the case of *Ps. sergenti*, blood-fed *Ph. sergenti* females after oviposition were used, whereas in the case of *Ps. tobbi*, oocysts were acquired from 4 to 7 day-old *Ph. tobbi* males. The homogenate was filtered through gauze and centrifuged (1700*g*) for 5 min, the supernatant was discarded, and the pellet was re-dissolved in 200 μl of water. The oocysts were counted using a Bürker counting chamber. For each species tested, 10–15 non-infected gravid sand fly females were placed into a rearing pot and allowed to oviposit; the number of eggs was counted under a stereomicroscope and the amount of gregarine oocysts corresponding to an infectious dose of 50 oocysts per egg were added to the food given to 1st instar larvae. Fourth-instar larvae, emerged adults, and gravid females after a blood meal were examined for evidence of gregarine infection as described above. Two different forms of 4th instar larvae were distinguished: actively feeding with gut filled with larval diet (further referred as "before defecation") and those ready to pupate with a "milky" gut, because of defecated midgut content (further referred as "after defecation").

2.4. DNA extraction, PCR amplification, sequencing, and phylogenetic analysis of SSU rDNA

DNA was isolated from *Ps. tobbi* n. sp. mature gametocysts dissected from *Ph. tobbi* adults. The procedures of DNA extraction, PCR

Table 1
Design of experimental infections of larvae of five sand fly species by two newly described gregarines.

Gregarine species	Natural sand fly host	Experimentally infected sand fly host
Ps. sergenti n. sp.	*Ph. sergenti*[a] (Paraphlebotomus)	*Ph. sergenti* (Paraphlebotomus)[b] *Ph. papatasi* (Phlebotomus) *Ph. tobbi* (Larroussius)[b] *Ph. arabicus* (Adlerius)
Ps. tobbi n. sp.	*Ph. tobbi*[a] (Larroussius)	*Ph. sergenti* (Paraphlebotomus)[b] *Ph. perniciosus* (Larroussius)

[a] Infected colony.
[b] Non-infected colony.

amplification, and sequencing of SSU rDNA were described by Votýpka et al. (2009). The primers used for sequencing were: the vector primers M13 (F) 5′-GTAAAACCACGGCCAG-3′ and M13 (R) 5′-CAGGAAACAGCTATGAC-3′ and internal primers oriented in both directions 5′-AAGACGATCAGATACCG-3′, 5′-TCGATTCCGGAGAGGGA-3′, 5′-CGTCAATTCCTTTAAG-3′, 5′-GCTGGCACCAGACTTGC-3′. The obtained SSU sequence of *Ps. tobbi* was deposited in GenBank under accessional number GQ329865. SSU rDNA sequences of *Ps. chagasi* and *Ps. sergenti* (marked as *Psychodiella* sp. from *Ph. sergenti*) originated from our previous study (Votýpka et al., 2009) and are deposited in GenBank under accessional numbers FJ865354 and FJ865355, respectively. Data set containing all gregarine sequences of small subunit ribosomal DNAs accessible at the time of the study was used to establish the phylogenetic position of both newly described species of sand fly gregarines. Phylogenetic analysis was performed following the same procedure as described previously by Votýpka et al. (2009).

3. Results

3.1. Description of morphology and life cycles

Different life stages of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi* are shown in Fig. 1. Their size characteristics and comparison are given in Tables 2 and 3.

3.1.1. Psychodiella sergenti n. sp.

Gamonts (Fig. 1A and J) are round or oval, aseptate, with distinct nucleus and nucleolus. Cytoplasm contains brown granules.

In young adults or when the infection intensity was high (over 30 gamonts per adult), some of the gamonts were smaller or prolonged. Gamonts were found in all examined stages of their host (4th instar larvae, adult males, and both, unfed and blood-fed females). Gamonts were mostly located in the intestine of 4th

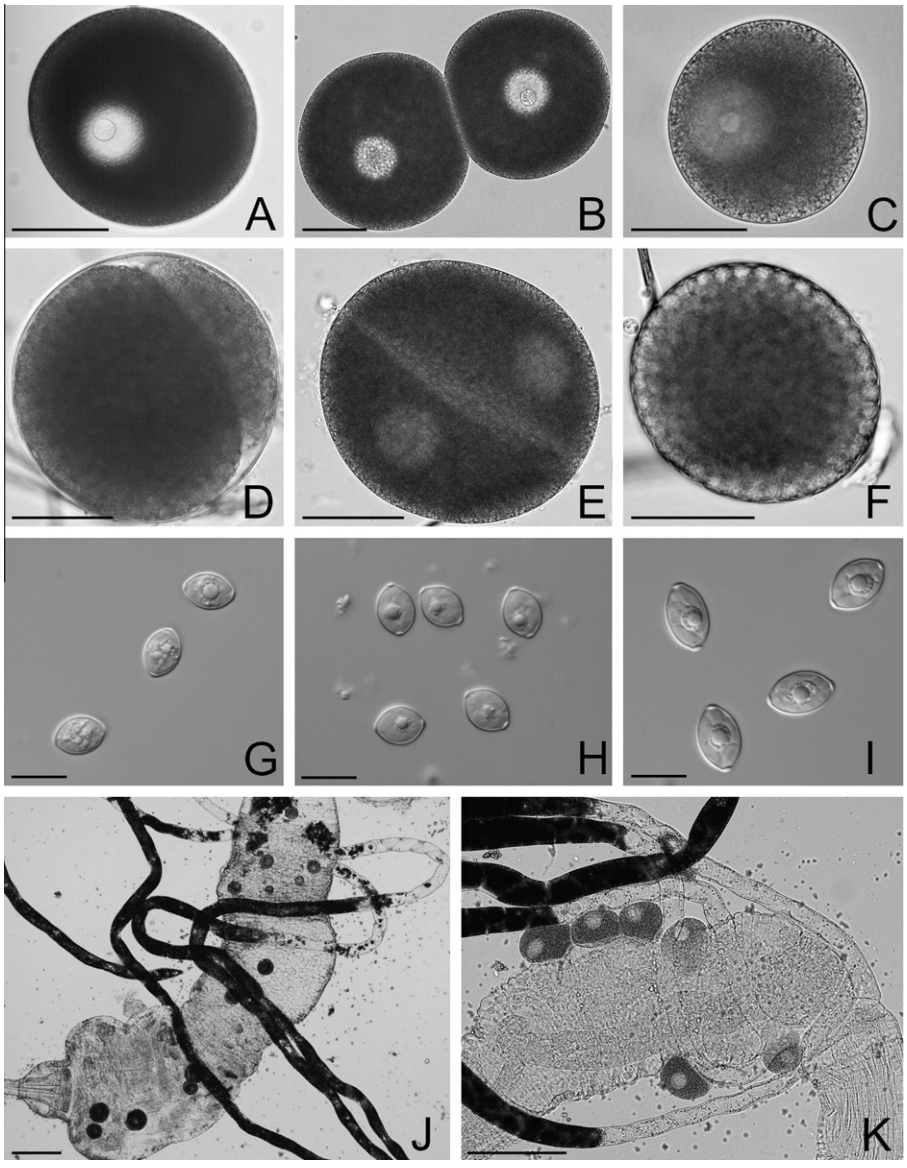

Fig. 1. Native preparations of different life cycle stages of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi*. Gamonts of *Ps. sergenti* (A), *Ps. tobbi* (B), and *Ps. chagasi* (C), scale bars = 50 μm; gametocysts of *Ps. sergenti* (D), *Ps. tobbi* (E), and *Ps. chagasi* (F), scale bars = 50 μm; oocysts of *Ps. sergenti* (G), *Ps. tobbi* (H), and *Ps. chagasi* (I) from the adult body cavity of appropriate sand fly species, scale bars = 10 μm. Gamonts of *Ps. sergenti* in the lumen of *Ph. sergenti* 4th instar larva after defecation (J), scale bar = 150 μm. Gamonts of *Ps. tobbi* outside the intestine of *Ph. tobbi* 4th instar larva after defecation (K), scale bar = 150 μm.

Table 2
Mean length of gamonts (T) and gametocysts (G) and length (O-L), width (O-W), and length/width ratio (O-LW) of oocysts (all in μm) with basic statistical characteristics of the three different gregarines from adults of *Phlebotomus sergenti*, *Ph. tobbi*, and *Lutzomyia longipalpis*.

Stage/species	*N*	Mean	Median	SD	Min.	Max.
T						
Ps. sergenti	408	114.6	112.8	21.5	59.0	188.0
Ps. tobbi	188	123.6	123.0	27.2	54.5	204.1
Ps. chagasi	133	80.6	84.0	21.9	25.5	168.0
G						
Ps. sergenti	86	128.2	131.5	17.3	84.6	169.2
Ps. tobbi	77	137.2	132.6	22.0	102.6	189.0
Ps. chagasi	58	105.0	103.4	14.7	84.0	150.0
O-L						
Ps. sergenti	231	9.6	9.6	0.3	8.7	10.3
Ps. tobbi	194	9.6	9.6	0.3	8.8	10.7
Ps. chagasi	113	12.7	12.7	0.3	12.0	13.3
O-W						
Ps. sergenti	231	6.7	6.7	0.2	6.2	7.1
Ps. tobbi	194	7.5	7.5	0.3	6.8	8.5
Ps. chagasi	113	8.3	8.3	0.3	7.3	8.9
O-LW						
Ps. sergenti	231	1.44	1.44	0.05	1.30	1.56
Ps. tobbi	194	1.28	1.28	0.04	1.19	1.40
Ps. chagasi	113	1.52	1.52	0.06	1.41	1.68

Table 3
Size comparison among different stages of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi*. Stages: T = gamont; G = gametocyst; O = oocyst. Measurements: L = length; W = width; LW = length/width ratio. Gregarines: PS = *Ps. sergenti* n. sp.; PT = *Ps. tobbi* n. sp.; PC = *Ps. chagasi*.

Stage/species	*t*-Value	D.f.	*P*	Comparison
T				
PS–PC	15.75	539	<0.01	PS > PC
PT–PC	15.12	319	<0.01	PT > PC
PT–PS	4.37	594	<0.01	PT > PS
G				
PS–PC	8.36	142	<0.01	PS > PC
PT–PC	9.63	133	<0.01	PT > PC
PT–PS	2.93	161	<0.01	PT > PS
O-L				
PS–PC	99.67	342	<0.01	PS < PC
PT–PC	84.25	305	<0.01	PT < PC
PT–PS	−0.39	423	>0.05	PT ≈ PS
O-W				
PS–PC	70.70	342	<0.01	PS < PC
PT–PC	27.81	305	<0.01	PT < PC
PT–PS	−38.42	423	<0.01	PT > PS
O-LW				
PS–PC	15.09	342	<0.01	PS < PC
PT–PC	44.40	305	<0.01	PT < PC
PT–PS	37.21	423	<0.01	PT < PS

instar larvae and in the body cavity of adults. In one larva, gamonts were found in the body cavity and in two adults in the fat body. In larvae before defecation, gamonts (as well as gametocysts) were situated more often in the posterior part of the midgut, usually in the ectoperitrophic space. However, in larvae after defecation, gamonts were located within the full length of the midgut lumen (Fig. 1J).

Gametocysts (Fig. 1D) have a distinct wall and are usually round or oval. Young gametocysts are formed by two gamonts with visible nuclei. Older gametocysts are filled with granules that suggest sporogony.

Gametocysts were only found in the midgut of 4th instar larvae and in blood-fed females, where they were attached to the accessory glands or lying freely in the body cavity. No sexual development occurred in males or unfed females.

Oocysts (Fig. 1G) are broad spindle-shaped (hesperidiform) with flattened, rather indistinctive ends, containing eight sporozoites. The length/width ratio is 1.44 ± 0.05 (range: 1.30–1.56).

They were observed exclusively in blood-fed females. During dissection, some gametocysts ruptured, adopting a characteristic horseshoe-like shape and releasing oocysts into the body cavity. In several blood-fed females, oocysts were observed in the lumen of accessory glands.

3.1.2. Psychodiella tobbi n. sp

Gamonts (Fig. 1B and K) similar to *Ps. sergenti* gamonts.

Gamonts were found in the body cavity of all examined adult sand fly stages. In 4th instar larvae before defecation, gamonts were usually located in the ectoperitrophic space of the hindgut of younger individuals and/or the body cavity of older individuals. In larvae after defecation, they were usually found in the body cavity and occasionally in the intestine (Fig. 1K).

Gametocysts (Fig. 1E) are round or oval, brownish, with a distinct wall.

Gametocysts were located in the intestine, the ectoperitrophic space of the hindgut, or the body cavity of 4th instar larvae. In adults, they were found in the body cavity. Sexual development of gregarines appeared 1 day after emergence in females and 4 days after emergence in males and it was not induced by a blood meal in the females.

Oocysts (Fig. 1H) are broad spindle-shaped (hesperidiform) with distinctive "button-like" ends, containing eight sporozoites. The length/width ratio is 1.28 ± 0.04 (range: 1.19–1.40).

Oocysts with eight sporozoites were only found in the body cavity of adults and in the lumen of accessory glands of females. Both gametocysts and oocysts appeared earlier in females than in males.

3.2. Size differences among stages of Psychodiella species

The size and differences between stages of the three studied gregarine species (*Ps. sergenti*, *Ps. tobbi*, and *Ps. chagasi*) are presented in Tables 2 and 3, and Fig. 2.

Both Old World species, *Ps. sergenti* and *Ps. tobbi*, had significantly bigger gamonts (114.6/123.6 μm > 80.6 μm; $P < 0.01$) and gametocysts (128.2/137.2 μm > 105.0 μm; $P < 0.01$) and significantly shorter oocysts (9.6/9.6 μm < 12.7 μm; $P < 0.01$) than the New World species *Ps. chagasi*. *Ps. tobbi* had significantly bigger gamonts (123.6 > 114.6 μm; $P < 0.01$) and gametocysts (137.2 > 128.2 μm; $P < 0.01$) than *Ps. sergenti*. The difference in the length of oocysts between *Ps. tobbi* and *Ps. sergenti* was not significant (9.6 μm and 9.6 μm; $P > 0.05$).

All three gregarine species significantly ($P < 0.01$) varied in the width of oocysts from each other with *Ps. chagasi* having the widest ones (8.3 μm), *Ps. tobbi* with 7.5 μm wide oocysts and *Ps. sergenti* possessing the narrowest oocysts of 6.7 μm. In order to sufficiently distinguish between *Ps. sergenti* and *Ps. tobbi* oocysts, a length/width ratio was evaluated. *Ps. sergenti* length/width ratio was significantly bigger than the one of *Ps. tobbi* (1.44 > 1.28; $P < 0.01$). Graphical representation of individual oocyst measurements is shown in Fig. 2.

3.3. Prevalence in wild caught sand flies

The prevalence in wild caught *Ph. sergenti* was 15 gregarine infected females out of 96 and in wild caught *Ph. tobbi* was 21 out of 125 females.

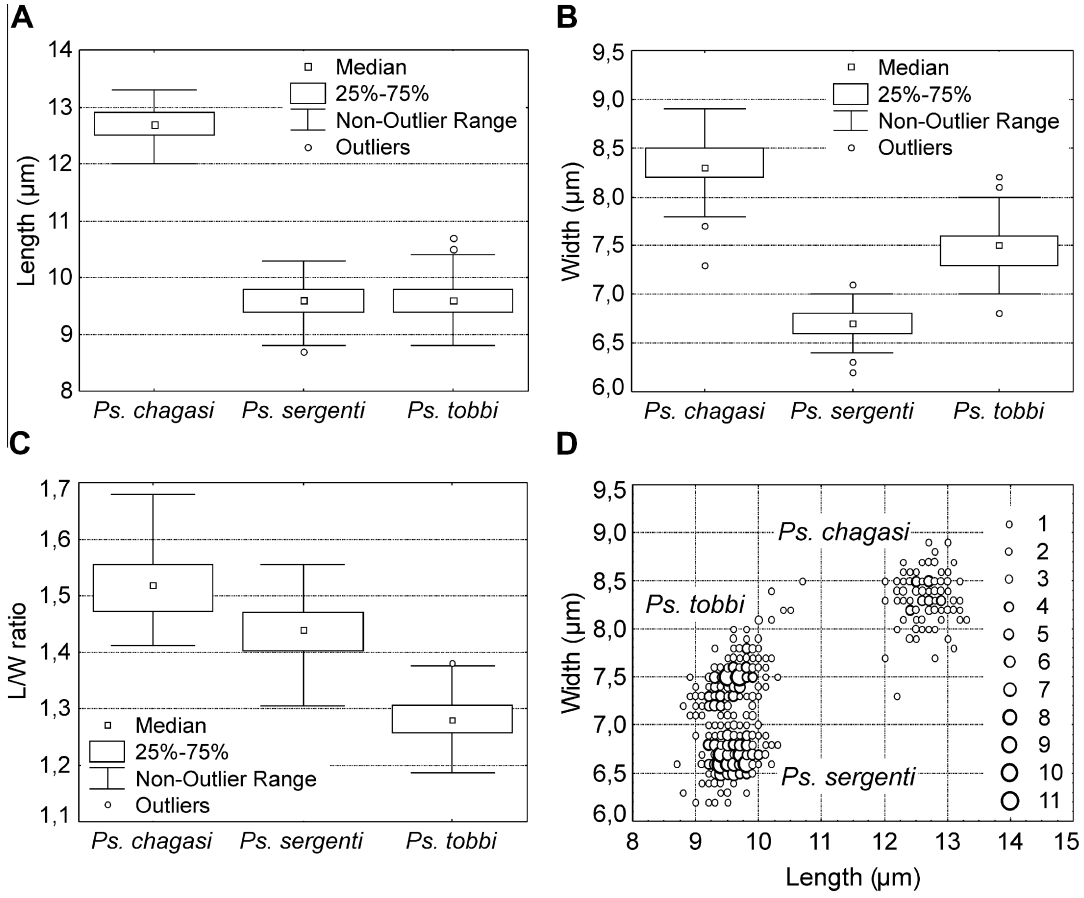

Fig. 2. Characteristics of *Psychodiella sergenti* n. sp., *Ps. tobbi* n. sp., and *Ps. chagasi* oocysts: length (A); width (B); length/width ratio (C); frequency scatterplot of oocyst measurements (D).

Table 4
Presence of *Psychodiella sergenti* n. sp. stages in four experimentally infected sand fly species. L4 = 4th instar larvae; M = males; F = females; FB = blood-fed females; T = gamonts; G = gametocysts; O = oocysts; + = gregarine found; (+) = gregarine found in low number and in less than 10% of individuals; – = gregarine not found.

	Ph. sergenti				*Ph. papatasi*				*Ph. arabicus*				*Ph. tobbi*			
	L4	M	F	FB	L4	M	F	FB	L4	M	F	FB	L4	M	F	FB
T	+	+	+	+	+	+	(+)	(+)	–	–	–	–	+	+	+	+
G	+	–	–	+	+	–	–	(+)	–	–	–	–	–	–	–	–
O	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	(+)

3.4. Experimental infection of various sand fly species

Testing four sand fly species belonging to different subgenera, the gregarine *Ps. sergenti* was able to complete the life cycle and fully develop only in its natural host, *Ph. sergenti* (Table 4 and Fig. 3). No infection occurred in any of the stages of *Ph. arabicus*. In *Ph. papatasi*, gamonts were found in all life cycle stages, gametocysts were found in 4th instar larvae and blood-fed females, but oocysts were not found in any of the stages. *Ph. tobbi* was also fairly refractory to *Ps. sergenti* infection. No gametocysts were found in any of the studied *Ph. tobbi* stages and few oocysts were found only in the body cavity of one blood-fed female out of 76 dissected. The number of gamonts per individual of *Ph. tobbi* was usually one or two, unlike in its natural host *Ph. sergenti*, where the intensity of infection was about three to 20 gamonts per individual.

Testing two sand fly species, *Ps. tobbi* was not able to mature and produce oocysts in *Ph. sergenti* (Table 5 and Fig. 4). No gregarine infection occurred in 4th instar larvae or unfed and blood-fed females. Out of 120 dissected *Ph. sergenti* males, only two carried a single gamont of *Ps. tobbi*. The life cycle of *Ps. tobbi* in *Ph. perniciosus* was similar to the one seen in *Ph. tobbi*; sexual development was not induced by blood meal of females and occurred also in males and unfed females. In *Ph. perniciosus*, oocysts were found in the lumen of accessory glands of only two females out of 176; gamonts and gametocysts were found in low numbers (1–4) in adults and 4th instar larvae.

3.5. Phylogenetic analysis

We sequenced 1752 base pairs of the SSU rRNA gene of the new *Psychodiella* species from *Ph. tobbi*. The final data set contained all

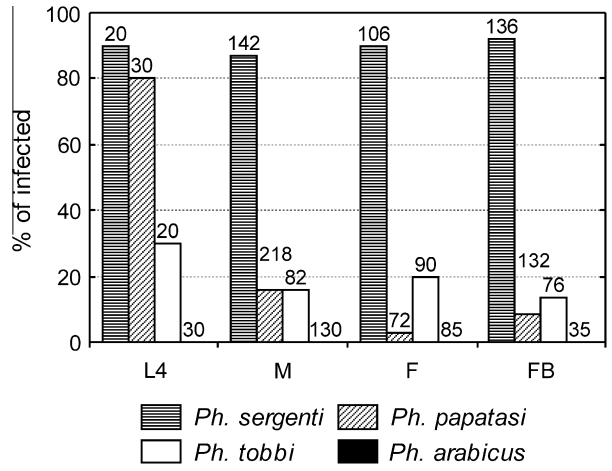

Fig. 3. Proportion of infected sand flies (*Phlebotomus sergenti*, *Ph. papatasi*, *Ph. tobbi*, and *Ph. arabicus*) with *Psychodiella sergenti* n. sp. Numbers above the columns represent the number of examined individuals.

Table 5
Presence of *Psychodiella tobbi* n. sp. stages in two experimentally infected sand fly species. L4 = 4th instar larvae; M = males; F = females; FB = blood-fed females; T = gamonts; G = gametocysts; O = oocysts; + = gregarine found; (+) = gregarine found in low number and in less than 10% of individuals; – = gregarine not found.

	Ph. sergenti				*Ph. perniciosus*			
	L4	M	F	FB	L4	M	F	FB
T	–	(+)	–	–	+	+	+	+
G	–	–	–	–	(+)	–	(+)	–
O	–	–	–	–	–	–	(+)	(+)

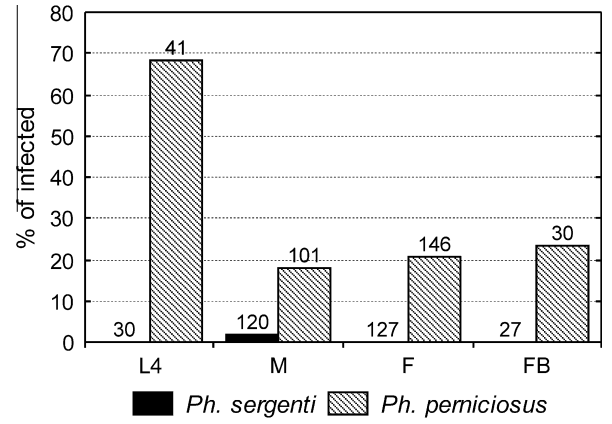

Fig. 4. Proportion of infected sand flies (*Phlebotomus sergenti* and *Ph. perniciosus*) with *Psychodiella tobbi* n. sp. Numbers above the columns represent the number of examined individuals.

sequenced species of named gregarines including the *Ps. sergenti* sequence from the previous work of Votýpka et al. (2009). The tree was rooted using cryptosporids as an outgroup and all major branches were well supported (data not shown) and correspond with the phylogenetic tree recently published by Votýpka et al. (2009). The tree revealed the relationship of both newly described gregarines (*Ps. sergenti* and *Ps. tobbi*) and *Ps. chagasi* and the monophyly of a separate genus *Psychodiella* is supported by high bootstrap values for all methods used (MP, 100%; ML, 100%; BA, 1.00; Fig. 5). The Old World sand fly gregarines, *Ps. sergenti* and *Ps. tobbi*, are closer to each other (genetic uncorrected p-distance was 1%, 17 nucleotide changes) than to the New World *Ps. chagasi* (2%, 35 and 30 changes, respectively).

4. Discussion

The life cycle of *Ps. sergenti* n. sp. differs markedly from *Ps. chagasi*. In adult *Ph. sergenti*, mature oocysts occur only in blood-fed females, unlike *Ps. chagasi*, whose sexual development occurs also in males and unfed females. Morphological differences as well as molecular phylogenetic analysis bring further evidence that *Ps. sergenti* n. sp. and *Ps. tobbi* n. sp. are different from *Ps. chagasi*. Broad-spindle oocysts with wider midsections of both newly described species differ from longer oocysts of *Ps. saraviae* that have narrower midsections and thicker walls (Ostrovska et al., 1990).

The only Old World sand fly gregarine species described previously, *Ps. mackiei*, was found in larvae, pupae, and adults of *Ph. argentipes* and *Ph. papatasi*, respectively (Shortt and Swaminath, 1927; Missiroli, 1929, 1932). It is the only sand fly gregarine where intracellular stages in the larval gut epithelium were observed. Previously reported observations that the sexual cycle of *Ps. mackiei* occurs in males and unfed females, that gametocysts attach to oviducts when oocysts are injected into the lumen, and the differences in the size of the gregarine stages provide strong evidence that *Ps. sergenti* and *Ps. tobbi* are well differentiated from *Ps. mackiei*.

Ps. sergenti varies from *Ps. tobbi* in the size of gamonts and gametocysts, but not in the length of oocysts. However, oocysts of these two gregarines clearly differ in the width and length/width ratio, which is bigger for *Ps. sergenti* (1.44) compared to *Ps. tobbi* (1.28). The length/width ratio intersection of the two species is only 0.1 and using this feature, *Ps. sergenti* can be clearly distinguished from *Ps. tobbi*. The length/width ratio can then serve as an unambiguous species characteristic. Besides morphological and phylogenetic differences and strict host specificity, both newly described species *Ps. sergenti* and *Ps. tobbi* differ from each other in their life cycles. In adults, sexual development of *Ps. sergenti* occurs exclusively in females after a blood meal unlike *Ps. tobbi*, where development also takes place in males and unfed females. For *Ps. sergenti*, gamonts were found mostly in the intestinal lumen of larvae, while for *Ps. tobbi*, they were also found in the body cavity.

How gregarines get from the larval intestine to the body cavity of sand fly adults was discussed by Shortt and Swaminath (1927). The authors propose the hypothesis that gregarines passively enter into the body cavity of pupae during tissue reconstitution in the early stage of pupation. However, our results may suggest that gregarines actively leave the larval intestine since they are found in the hemocoel of older *Ph. tobbi* larvae before body reconstitution occurred. In *Ps. tobbi*, the "transfer" to the host body cavity occurs earlier in the larval development, while gregarines of *Ps. sergenti* are in the intestine of all larval stages and emerge later in the body cavity of pupae.

Not only the morphological and the life cycle differences, and the phylogenetic position of *Ps. tobbi* and *Ps. sergenti*, but also the inability of *Ps. tobbi* to produce gametocysts or oocysts in *Ph. sergenti* and very low susceptibility of *Ph. tobbi* to *Ps. sergenti* confirm that the two newly described gregarines are distinct species. The full development of *Ps. sergenti* and *Ps. tobbi* takes place only in their natural hosts. In preliminary studies on *Phlebotomus* (*Phlebotomus*) *duboscqi*, sand flies can become accidentally infected with *Ps. sergenti*, however in all cases the number of gregarines found in foreign hosts was substantially smaller than in their natural hosts. Figs. 3 and 4, showing the proportions of infected sand fly

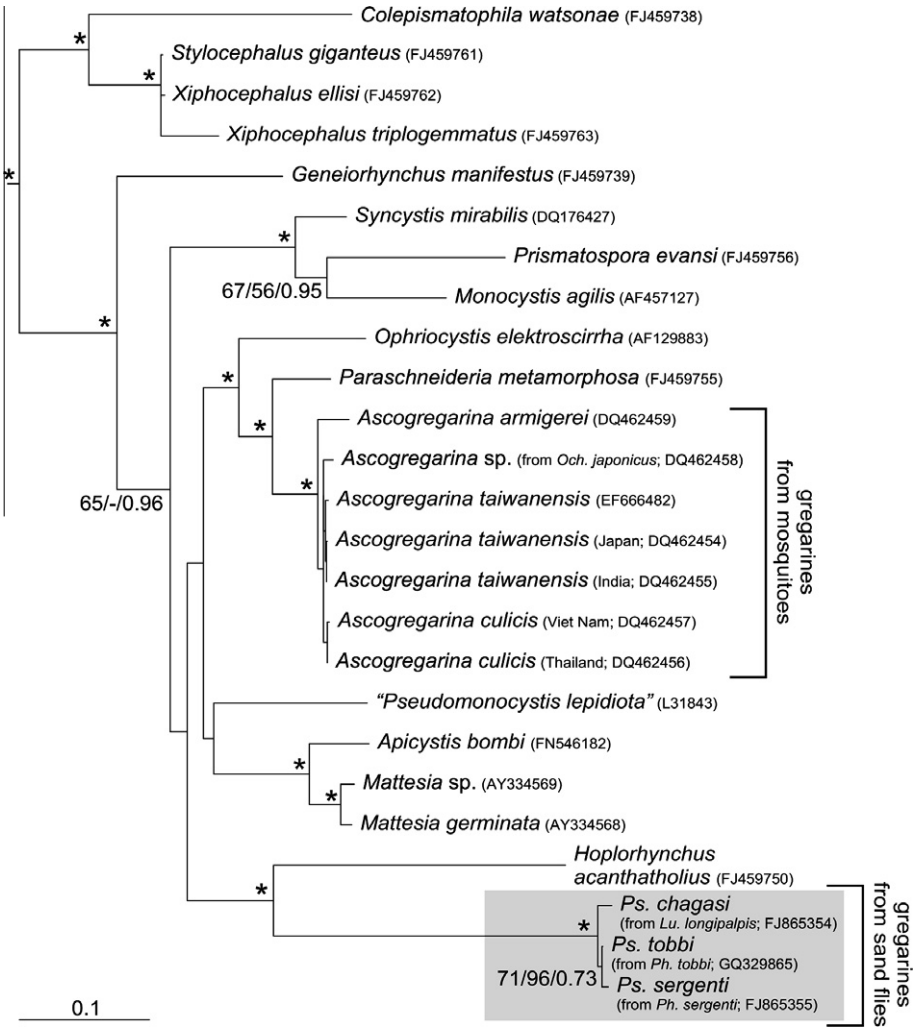

Fig. 5. Part of a maximum likelihood (ML) phylogenetic tree inferred from SSU rRNA gene sequences. The figure shows the topology obtained by maximum likelihood using the GTR model for nucleotide substitutions with Γ-distribution in 8 + 1 categories as implemented in PhyML. Bootstrap values from ML, MP (1000 replicates), and Bayesian posterior probabilities (number of generations was 5×10^6) are shown above branches. Asterisks (*) at the nodes denote Bayesian posterior probabilities and bootstrap percentages of 95% or higher. Bar represents 0.1 substitutions per site.

stages, suggest that the bottleneck for gregarines in an artificial host is larval development and pupation, because the percentage of infected sand flies decreases rapidly in adults. The results of cross-infections are in agreement with findings of Wu and Tesh (1989), but contradict studies demonstrating broad host range of gregarines from nematoceran Diptera (Shortt and Swaminath, 1927; Missiroli, 1929; Levine, 1977; Garcia et al., 1994). The conspecificity of the gregarine *Ps. mackiei* found in *Phlebotomus* (*Euphlebotomus*) *argentipes* (Shortt and Swaminath, 1927) and *Ph.* (*Phlebotomus*) *papatasi* (Missiroli, 1929) is also questioned, since the two phlebotomine genera do not form a monophyletic clade (Aransay et al., 2000).

Due to the differences in the life cycle, morphology, and dimensions of gamonts, gametocysts and particularly oocysts, and the results of cross-infection studies and molecular phylogenetic analysis, we clearly demonstrated that gregarines from *Ph. tobbi* and *Ph. sergenti* are two new species of the genus *Psychodiella*.

5. Taxonomic summary

Phylum: Apicomplexa Levine, 1970.
Order: Eugregarinorida Léger, 1900.
Suborder: Aseptatorina Chakravarty, 1960.
Genus: *Psychodiella* Votýpka, Lantová and Volf, 2009.

5.1. *Psychodiella sergenti n. sp. Lantová, Volf and Votýpka*

Type specimens: *Ps. sergenti* n. sp.
Type host: *Ph. sergenti* (Diptera: Psychodidae)
Type locality (origin of the sand fly colony): Turkey, South-East Anatolia, SanliUrfa (37°11′N, 38°48′E)
Site of infection: Midgut of 4th instar larvae and body cavity of adults. Oocysts in the lumen of accessory glands and in the body cavity, exclusively in blood-fed females.
Type material: Slides (No. PsSerF2d.1B1.2007/01) stained with PAS reaction followed by Ehrlich's Hematoxylin have been deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic.
Etymology: The species name has been adopted from the species name of the host sand fly, *Ph. sergenti* named in honour of the doctors Edmond and Etienne Sergent.
Diagnosis in the native: Gamonts (114.6 ± 21.5 μm) round or oval, aseptate, with distinct nucleus and nucleolus. Gametocysts (128.2 ± 17.3 μm) round or oval, sometimes with original two gamonts, older gametocysts with "granules" suggesting sporogony. Oocysts (length: 9.6 ± 0.3 μm, 8.7–10.3 μm; width: 6.7 ± 0.2, 6.2–7.1 μm) broad spindle-shaped (hesperidiform) with flattened, rather indistinctive ends, containing eight sporozoites. The length/width ratio is 1.44 ± 0.05 (1.30–1.56).

5.2. Psychodiella tobbi n. sp. Lantová, Volf and Votýpka

Type specimens: *Ps. tobbi* n. sp.
Type host: *Ph. tobbi* (Diptera: Psychodidae)
Type locality (origin of the sand fly colony): Turkey, South Anatolia, Tepecikoren (37°36′N, 35°62′E)
Site of infection: Body cavity and intestine of 4th instar larvae, body cavity of adult sand flies. Oocysts in the body cavity and lumen of accessory glands of adult sand flies of both sexes.
Type material: Gregarine-infected *Ph. tobbi* males and females placed in a tube with AFA fixative (alcohol–formalin–acetic acid) (No. PsTobFM2d.2008/01) deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic.
Etymology: The species name has been adopted from the species name of the host sand fly, *Ph. tobbi* dedicated to Dr. Agha Khan Tobb.
Diagnosis in the native: Gamonts (123.6 ± 27.2 μm) round or oval, aseptate, with distinct nucleus and nucleolus. Gametocysts (137.2 ± 22 μm) round or oval, sometimes with original two gamonts, older gametocysts with granules suggesting sporogony. Oocysts (length: 9.6 ± 0.3 μm, 8.8–10.7 μm; width: 7.5 ± 0.3, 6.8–8.5 μm) broad spindle-shaped (hesperidiform) with distinctive "button-like" ends, containing eight sporozoites. The length/width ratio is 1.28 ± 0.04 (1.19–1.40).

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The Development of *Psychodiella sergenti* (Apicomplexa: Eugregarinorida) in *Phlebotomus sergenti* (Diptera: Psychodidae)

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Running title: Development of *Psychodiella sergenti*

SUMMARY

Introduction. *Psychodiella sergenti* is a recently described specific pathogen of the sand fly *Phlebotomus sergenti*, a major vector of *Leishmania tropica*. The aim of this study was to examine the life cycle of *Ps. sergenti* in various developmental stages of the sand fly host. **Methods.** The microscopical methods used include scanning electron microscopy, transmission electron microscopy and light microscopy of native preparations and histological sections stained with periodic acid-Schiff reaction. **Results.** *Psychodiella sergenti* oocysts were observed on the chorion of sand fly eggs. In 1st instar larvae, sporozoites were located in the ectoperitrophic space of the intestine. No intracellular stages were found. In 4th instar larvae, *Ps. sergenti* was mostly located in the ectoperitrophic space of the intestine of the larvae before defecation and in the intestinal lumen of the larvae after defecation. In adults, the parasite was recorded in the body cavity, where the sexual development was triggered by a blood meal intake. **Conclusion.** *Psychodiella sergenti* has several unique features. It develops sexually exclusively in sand fly females that took a blood meal, and its sporozoites bear a distinctive conoid, which is longer than conoids of the mosquito gregarines.

Key words: *Psychodiella*, *Psychodiella sergenti*, Gregarine, *Phlebotomus sergenti*, Sand fly, Life cycle, PAS, Egg, Larva, Adult

INTRODUCTION

Gregarines parasitizing sand flies (Apicomplexa: Eugregarinorida) are aseptate eugregarines recently separated from the mosquito genus *Ascogregarina* Ward, Levine and Craig, 1982 to form a new genus *Psychodiella* Votypka, Lantova and Volf, 2009 (Votypka et al. 2009). Despite their high host specificity, only five *Psychodiella* species have been described so far. Numerous studies on the mosquito *Ascogregarina* species (e.g. Walsh and Callaway, 1969; Vavra, 1969; Sanders and Poinar, 1973; Munstermann and Levine, 1983; Chen et al. 1997a) showed that mosquito gregarines differ from sand fly gregarines at two critical points of the life cycle: in mosquito larvae, the gregarines develop intracellularly in the intestinal epithelial cells and in adults, they are located in the Malpighian tubules.

Psychodiella life cycle has been studied in detail in *Psychodiella chagasi* (Adler and Mayrink, 1961) by Adler and Mayrink (1961), Coelho and Falcao (1964) or Warburg and Ostrovska (1991). Briefly: the first instar larvae are infected by swallowing the gregarine oocysts. Sporozoites released from oocysts reside in the larval midgut and develop into trophozoites. Later, gamonts can be found mostly in the gut lumen of larvae, where the gregarines undergo sexual development from the formation of syzygies to the production of oocysts. In adults, the gregarines are located in the body cavity forming syzygies and gametocysts with oocysts inside. In females, gametocysts attach to accessory glands, and oocysts are injected into their lumen. During oviposition, contents of the glands including the oocysts are attached to the chorion of eggs and serve as the source of infection for newly hatched larvae. This general life cycle is modified in other *Psychodiella* species, and differences were described in *Psychodiella mackiei* (Shortt and Swaminath, 1927) and *Psychodiella tobbi* Lantova, Volf and Votypka, 2010.

Psychodiella sergenti Lantova, Volf and Votypka, 2010 is a recently described specific pathogen of the sand fly *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae), an important vector of *Leishmania tropica* (Wright, 1903) (e.g. Killick-Kendrick et al. 1995), which is a causative agent of human cutaneous leishmaniasis. Native preparations of sand fly adults revealed that sexual development of *Ps. sergenti* (formation of syzygies, gametocysts and oocysts) occurs only in female sand flies and is conditioned by a blood meal

intake (Lantova et al. 2010). The aim of the present study was to document *Ps. sergenti* life cycle in various life stages of its host including the eggs and 1st instar larvae. Several microscopical methods were used: scanning electron microscopy, transmission electron microscopy and light microscopy of native preparations and histological sections stained with PAS reaction (periodic acid-Schiff). Using such a wide variety of microscopical techniques gives a clear overview of the whole parasite's life cycle.

MATERIALS AND METHODS

Sand flies

Phlebotomus sergenti colony infected with *Ps. sergenti* originated from females collected in Sanli Urfa, Turkey. The colony maintenance was described by Volf and Volfova (2011) and included an egg-washing procedure using a series of solutions (Poinar and Thomas, 1984) to reduce the infection intensity of this pathogenic gregarine (Lantova, unpublished observations). The washing was omitted in a batch of eggs used for this study.

Native preparations

Adults of both sexes and different ages were used, as well as two groups of 4th instar larvae: those with gut filled with larval diet, further referred to as larvae before defecation (BD) and those ready to pupate with an empty gut, further referred to as larvae after defecation (AD). The specimens were immobilized on ice and dissected in phosphate-buffered saline (PBS) under a stereomicroscope SZX-12 (Olympus Corporation, Tokyo, Japan). Micrographs were produced with DP-70 digital camera (Olympus) connected to an optical microscope BX-51 (Olympus).

Scanning microscopy

Gravid sand fly females were left to oviposit into a plastic cup filled with plaster (commonly used for the colony, see Volf and Volfova, 2011), and one to two days later, the eggs were transferred using a fine brush onto double-sided tape. Oocysts were documented on the surface of *Ph. sergenti* eggs using a scanning microscope TM-1000 (Hitachi High-Technologies Corporation, Tokyo,

Japan). This scanning microscope does not require any sample preparation and allows direct observation of unfixed and non-desiccated samples.

Electron microscopy

Three-day old larvae of 1st instar were fixed in modified Karnovsky fixative (Karnovsky, 1965) or in 2.5% glutaraldehyde in cacodylate buffer (4°C), post-fixed in 2% osmium tetroxide in cacodylate buffer (4°C), dehydrated through an ascending ethanol and acetone series and embedded in Araldite 502/PolyBed 812 (Polysciences Inc., Warrington, PA, USA). Thin sections (70 nm) were prepared on a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and stained using uranyl acetate and lead citrate (Reynolds, 1963). The sections were examined and photographed using an electron microscope JEM-1011 (JEOL Ltd., Tokyo, Japan).

Histology

Larvae of 4th instar (BD and AD) and adults at different days post eclosion were dissected and fixed at 4°C in AFA fixative (96% ethanol: 38% formaldehyde: acetic acid: distilled water; 12.5: 1.5: 1: 10). The head and the last posterior segments were removed for better penetration of fixatives. Specimens were then washed three times in PBS and 70% ethanol and embedded in 2-hydroxyethyl methacrylate (JB-4 Plus Embedding Kit, Polysciences) following the manufacturer's instructions. Histological sections (2 – 4 µm) were prepared using a Shandon Finesse ME+ microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stained using PAS (periodic acid-Schiff) reaction with Ehrlich's acid hematoxylin: oxidization in 1% periodic acid for 5 min, Schiff's reagent (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 28 min, Ehrlich's acid hematoxylin for 4 min (with extensive washing in running water between steps). Stained sections were mounted on glass slides with Plastic UV Mount Mounting Media (Polysciences) and observed and photographed under an optical microscope BX-51 (Olympus) connected to a DP-70 digital camera (Olympus).

RESULTS

Ps. sergenti oocysts on sand fly eggs

The exochorion of *Ph. sergenti* eggs was contaminated with *Ps. sergenti* oocysts (Fig. 1). The number of oocysts per egg varied; some eggs appeared without any contamination, others contained dozens of oocysts. The distribution of oocysts on the chorion was not even, they usually concentrated around the tip of the egg (Fig. 1B, C) or along the longitudinal axis of the egg in contact with the exochorion sculpturing ridges (Fig. 1A, B). Manipulation with eggs by a brush occasionally caused detachment of the oocysts from the egg surface. In some cases, distinct imprints of the detached oocysts were visible (Fig. 1D)

Ps. sergenti sporozoites in 1st instar sand fly larvae

In 1st instar larvae, *Ps. sergenti* sporozoites were found in the intestine (Fig. 2). They were located mostly in the posterior midgut, exclusively in the ectoperitrophic space between the peritrophic matrix and the epithelium (Fig. 2A). The sporozoites were either crawling on microvilli or attached to the epithelial cells with a distinct mucron (Fig. 2B, E). They had a three-layered pellicle (Fig. 2C) and posteriorly located nucleus (Fig. 2E). A very distinct conoid as well as polar ring and numerous micronemes were observed (Fig. 2D, E). No intracellular stages were observed.

Ps. sergenti development in 4th instar sand fly larvae

The documentation of *Ps. sergenti* in 4th instar larvae was accomplished in the native preparations (Fig. 3A, B) and in the histological sections stained with PAS reaction with Ehrlich's acid hematoxylin (Fig. 3C, D).

Gregarine stages found in 4th instar larvae were mostly gamonts, occasionally also syzygies and gametocysts but never oocysts. In BD larvae, gamonts were documented in the ectoperitrophic space of the intestine, mainly in the posterior midgut (Fig. 3A, C), while in AD larvae, the gregarines were located in the midgut lumen along its whole length (Fig. 3B, D). In a few cases, gamonts were found also in the larval body cavity, but no intracellular development or cell damage was detected.

The PAS reaction proved to be very useful in highlighting gregarines in the host tissues. Their PAS-positive amylopectin granulation was typical and

easily recognizable from other PAS-positive objects, e.g. from the midgut contents (Fig. 3C).

Ps. sergenti development in sand fly males

Even though males up to 13 days post eclosion were examined, neither native observations nor histological sections recorded other gregarine life stages than gamonts (Fig. 4A – C). The gamonts were usually round or oval, but in high-intensity infections, some had a shape of an hourglass or a tear-drop. They were found mostly in the body cavity, in several cases also in the fat body but never in the intestine or elsewhere. A characteristic appearance of the gregarines with a distinctive nucleus (Fig. 4B) allowed distinguishing them easily from a rectal papilla, the PAS-positive tissue of adult sand flies (Fig. 4A).

Ps. sergenti development in sand fly females

In females that did not take a blood meal, no other gregarine stages but gamonts were found, even thought the females were dissected up to the age of 13 days. On the other hand, in blood-fed females, the whole sexual development including syzygies, gametocysts and oocysts was demonstrated (Figs. 4D – F and 5). Gamonts and gametocysts were located in the body cavity and a few gamonts, particularly when the infection intensity was high, in the fat body.

In females two days post blood meal, the young gametocysts were found still consisting of the two original gamonts with their nuclei (Fig. 5A). From three to five days after a blood meal, gametocysts at different stages of maturation were documented (Fig. 5D, E), some of them being attached to the accessory glands (Figs. 4D and 5B – E). Around day five, the gametocysts were fully matured, and accessory glands of blood-fed females became filled with oocysts (Figs. 4 E, F and 5 F – H).

In females, other strongly PAS-positive tissues are the rectal papilla (Fig. 5A) and oocytes (Fig. 5D – F); however, gregarines stained with PAS reaction were distinct, particularly in sections not post-stained with Ehrlich's acid hematoxylin (Fig. 5B, C).

DISCUSSION

The main distinctive feature of *Ps. sergenti* life cycle is the fact that the gregarines develop sexually exclusively in adult females that had a blood meal. This is a striking contrast to *Ps. chagasi* and *Ps. tobbi* where gametocysts are formed in adults of both sexes, including females that did not take a blood meal (Coelho and Falcao, 1964; Lantova et al. 2010). In *Ps. chagasi*, Coelho and Falcao (1964) and Warburg and Ostrovska (1991) found gregarine oocysts in 4th instar larvae, the former authors even described the formation of oocysts that were being defecated and served as a source of horizontal infection to other larvae. Contrastingly, no oocysts were found in the 4th instar larvae of *Ps. tobbi* (Lantova et al. 2010) or *Ps. sergenti* in this study.

The only other sand fly gregarine with the life cycle in adult hosts similar to *Ps. sergenti* is an undescribed parasite reported by Ayala (1971) in *Lutzomyia vexatrix occidentis* Fairchild and Hertig, 1957. Similarly to Ayala (1973) we hypothesize that the hormonal changes influenced by a blood meal intake trigger the gregarine's sexual cycle. Such behaviour is advantageous for the gregarines as they only invest energy to sexual development where the vertical transmission is expected – in blood-fed females.

Psychodiella sergenti sporozoites were found in 1st instar larvae either crawling on microvilli or attached to the epithelial cells with a mucron. No intracellular development of *Ps. sergenti* was recorded in the sand fly larvae. This is in agreement with findings of various authors on *Ps. chagasi* but in contrast to findings of Shortt and Swaminath (1927) on *Psychodiella mackiei* where intracellular stages were reported in the gut of 1st larvae.

Sporozoites of *Ps. sergenti* possess a long conoid similar to that of *Ps. chagasi* (Warburg and Ostrovska, 1991) but longer than those described in *Ascogregarina* species (e.g. Sheffield et al. 1971; Chen et al. 1997b). This suggests that long conoids are typical for *Psychodiella* gregarines. The surface of *Ps. sergenti s*porozoites consists of a three-layered pellicle. Previously, a two-layered pellicle was reported by Warburg and Ostrovska (1991) in *Ps. chagasi* and by Sheffield et al. (1971) and Sanders and Poinar (1973) in mosquito gregarine species. As pointed out by Vavra (1969), such a difference might be due to the fact that the two inner membranes could sometimes be very close giving the impression of a single membrane.

In 4th instar BD larvae, *Ps. sergenti* gamonts were located in the ectoperitrophic space. Similar results were reported for other *Psychodiella* species by Shortt and Swaminath (1927), Coelho and Falcao (1964), Warburg and Ostrovska (1991) or Lantova et al. (2010). The ectoperitrophic location protects the gregarines from being discharged when larvae before pupation defecate the whole gut content together with the peritrophic matrix.

In adults, the main location of the gregarines was the body cavity as also recorded for other species by Shortt and Swaminath (1927), Adler and Mayrink (1961) and Ostrovska et al. (1990). In high-intensity infections, we found *Ps. sergenti* also in the fat body. The attachment of the gametocysts to the accessory glands was recorded in all sand fly gregarine species (reviewed by Ostrovska et al. 1990) and provides an effective way of vertical transmission to the offspring.

Scanning electron microscopy showed *Ps. sergenti* oocysts frequently attached to the longitudinal exochorion sculpturing ridges of the sand fly eggs. In contrast, Adler and Mayrink (1961) recorded *Ps. chagasi* oocysts adhered to the *Lutzomyia longipalpis* (Lutz and Neiva, 1912) egg surface at a right angle to the longitudinal axis. This suggests that, similarly to the species-specific chorion ornamentation (e.g. Nogueira et al. 2004), also the character of oocyst-contamination might be species specific. The location of oocysts seems to be connected to the process of the exochorion formation during oviposition, when it is secreted by the accessory glands. The viscous consistency of the secretion enables the oocysts to adhere to the egg surface at the site where drying exochorion produces characteristic sculpturing ridges.

In general, *Psychodiella* life cycle has been studied in detail by a limited number of authors, and only a single work has been published documenting this parasite (*Ps. chagasi*) using electron microscopy (Warburg and Ostrovska, 1991). In the present study, we used various microscopical methods in major sand fly developmental stages giving a self-contained overview of *Ps. sergenti* life cycle.

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CAPTIONS TO FIGURES

Fig. 1. Scanning electron micrographs of *Phlebotomus sergenti* eggs infected with *Psychodiella sergenti*. (A – C) *Psychodiella sergenti* oocysts (arrows) attached to the chorion of eggs. (D) Imprints of the detached oocysts. Scale bars (A, B) = 50 μm, (C) = 25 μm, (D) = 10 μm.

Fig. 2. Transmission electron micrographs of the intestine of 1st instar *Phlebotomus sergenti* larvae infected with *Psychodiella sergenti*. (A) Unattached sporozoite (arrow) in the ectoperitrophic space of the intestine. (B, D, E) Sporozoites attached to the epithelial cells of the intestine. (C) Detail of a three-layered pellicle of the sporozoite. CO – conoid, IT – lumen of the larval intestine with organic debris and yeasts, MN – micronemes, MU – mucron, NU – nucleus, PM – peritrophic matrix, PR – polar ring. Scale bars (A) = 2 μm, (B, D, E) = 1 μm, (C) = 100 nm.

Fig. 3. Native preparations (A, B) and histological sections stained with PAS reaction and Ehrlich's acid hematoxylin (C, D) of *Phlebotomus sergenti* larvae of 4th instar infected with *Psychodiella sergenti*. (A, C) Intestine of 4th instar larva before defecation. The gregarines (arrows) are located in the ectoperitrophic space of the intestine. (B, D) Intestine of 4th instar larva after defecation. The gregarines (arrows) are located in the lumen of the intestine. FB – fat body, IT – intestine, MT – Malpighian tubules. Scale bars = 100 μm.

Fig. 4. Histological sections stained with PAS reaction and Ehrlich's acid hematoxylin of *Phlebotomus sergenti* males infected with *Psychodiella sergenti* (A, B) and native preparations of *Ph. sergenti* adults infected with *Ps. sergenti* (C – F). (A) Male body cavity filled with gamonts (arrows). (B) Gamonts with distinctive nuclei in the male body cavity (arrows). (C) Gamonts from the body cavity of a male sand fly. (D) Gametocyst (arrow) attached to the accessory gland of a female five days post blood meal. (E) Accessory gland of a female eight days post blood meal filled with oocysts. (F) Detail of oocysts. AG – accessory glands. IT – intestine, NG – neural ganglion, VS – vesicular seminalis, RP – rectal papilla. Scale bars (A, C – E) = 100 μm, (B) = 50 μm, (F) = 10 μm.

Fig. 5. Histological sections of *Phlebotomus sergenti* females two (A – C), three (D, E) and seven (F – H) days post blood meal infected with *Psychodiella sergenti* stained with PAS reaction and Ehrlich's acid hematoxylin. (A) Young gametocyst in the body cavity of a female two days post blood meal. (B) Gametocysts (arrows) attached to the accessory glands of a female two days post blood meal, stained only with PAS reaction. (C) Section E post-stained with Ehrlich's acid hematoxylin. (D) Gamonts and gametocysts (arrow) in the body cavity of a female three days post blood meal. (E) Gametocysts (arrows) at different stages of maturation attached to the accessory glands of a female three days post blood meal. (F) Female seven days post blood meal with gamonts (arrow) in the body cavity and oocysts in the accessory glands. (G) Accessory gland filled with gregarine oocysts. (H) Detail of oocysts. AG – accessory glands, IT – intestine, OC – oocytes, RP – rectal papilla. Scale bars (A, G) = 50 μm, (B - F) = 100 μm, (H) = 10 μm.

Fig. 1. Scanning electron micrographs of *Phlebotomus sergenti* eggs infected with *Psychodiella sergenti*.

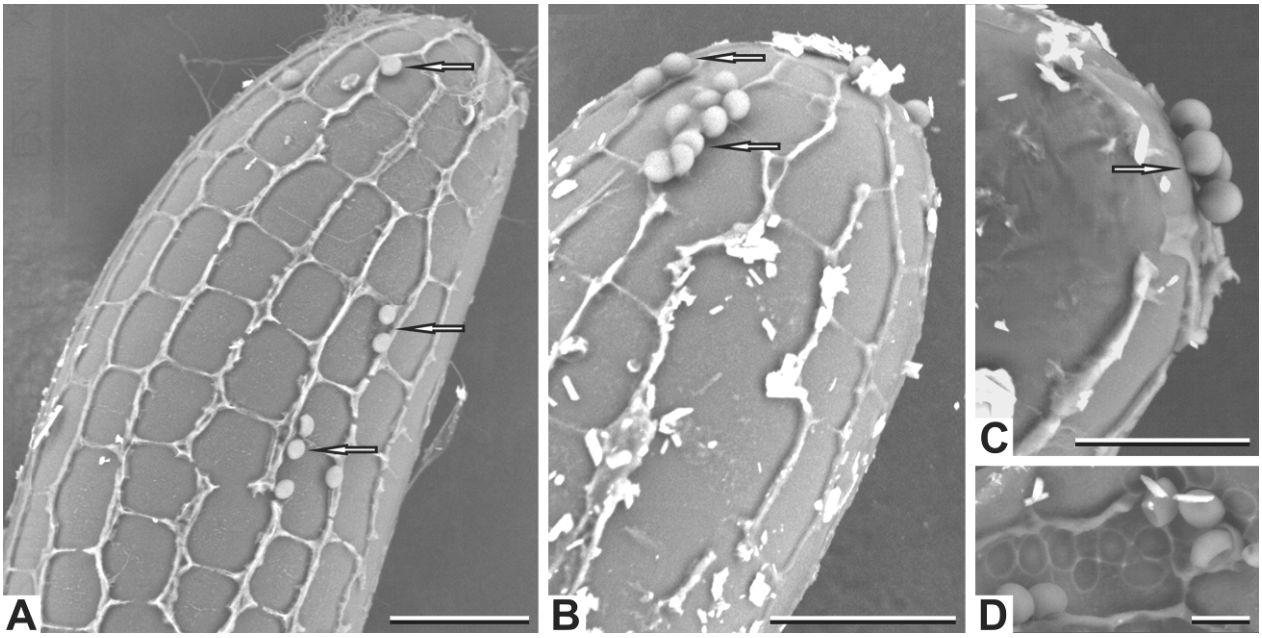


Fig. 2. Transmission electron micrographs of the intestine of 1st instar *Phlebotomus sergenti* larvae infected with *Psychodiella sergenti*.

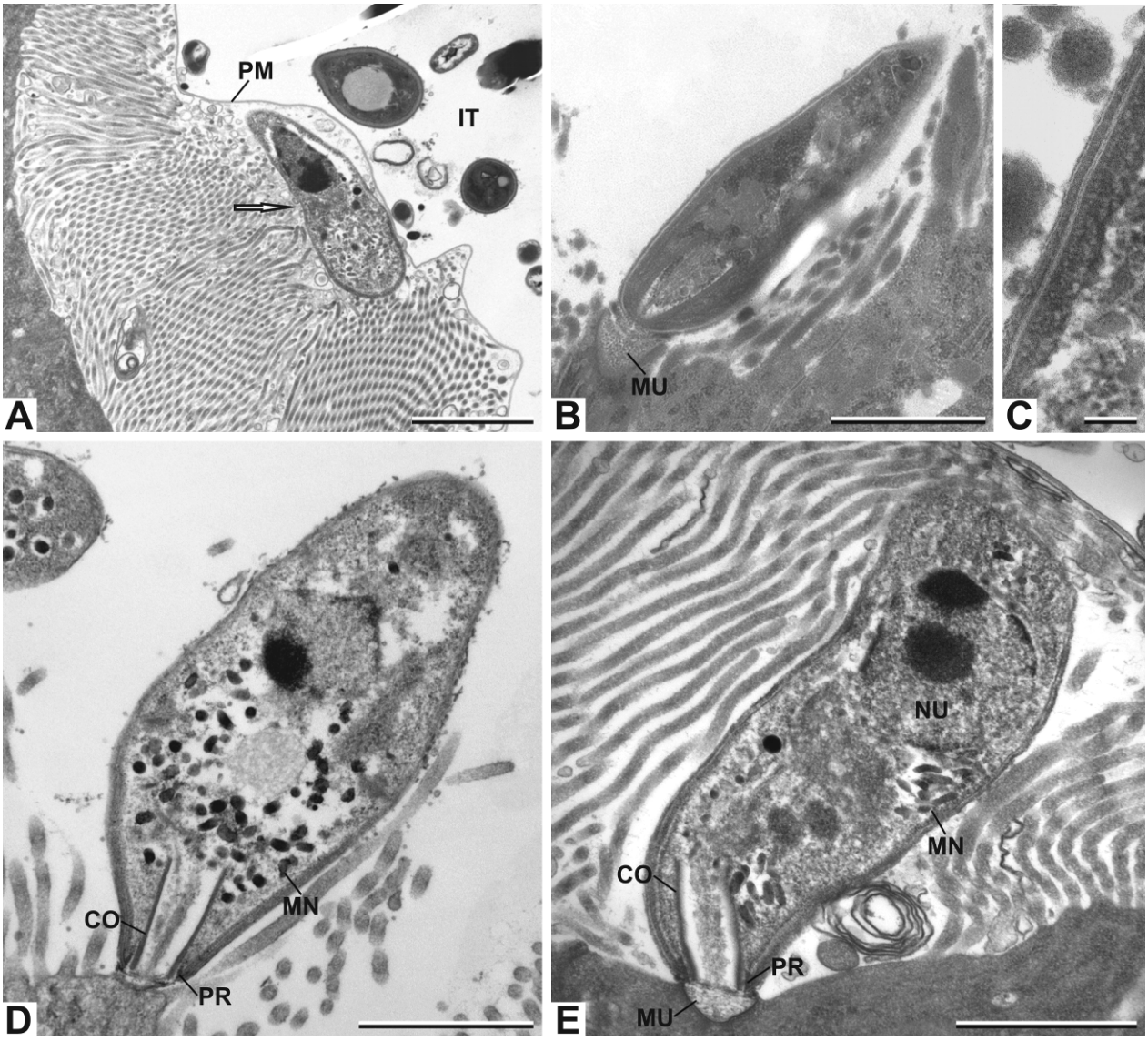


Fig. 3. Native preparations (A, B) and histological sections stained with PAS reaction and Ehrlich's acid hematoxylin (C, D) of *Phlebotomus sergenti* larvae of 4th instar infected with *Psychodiella sergenti*.

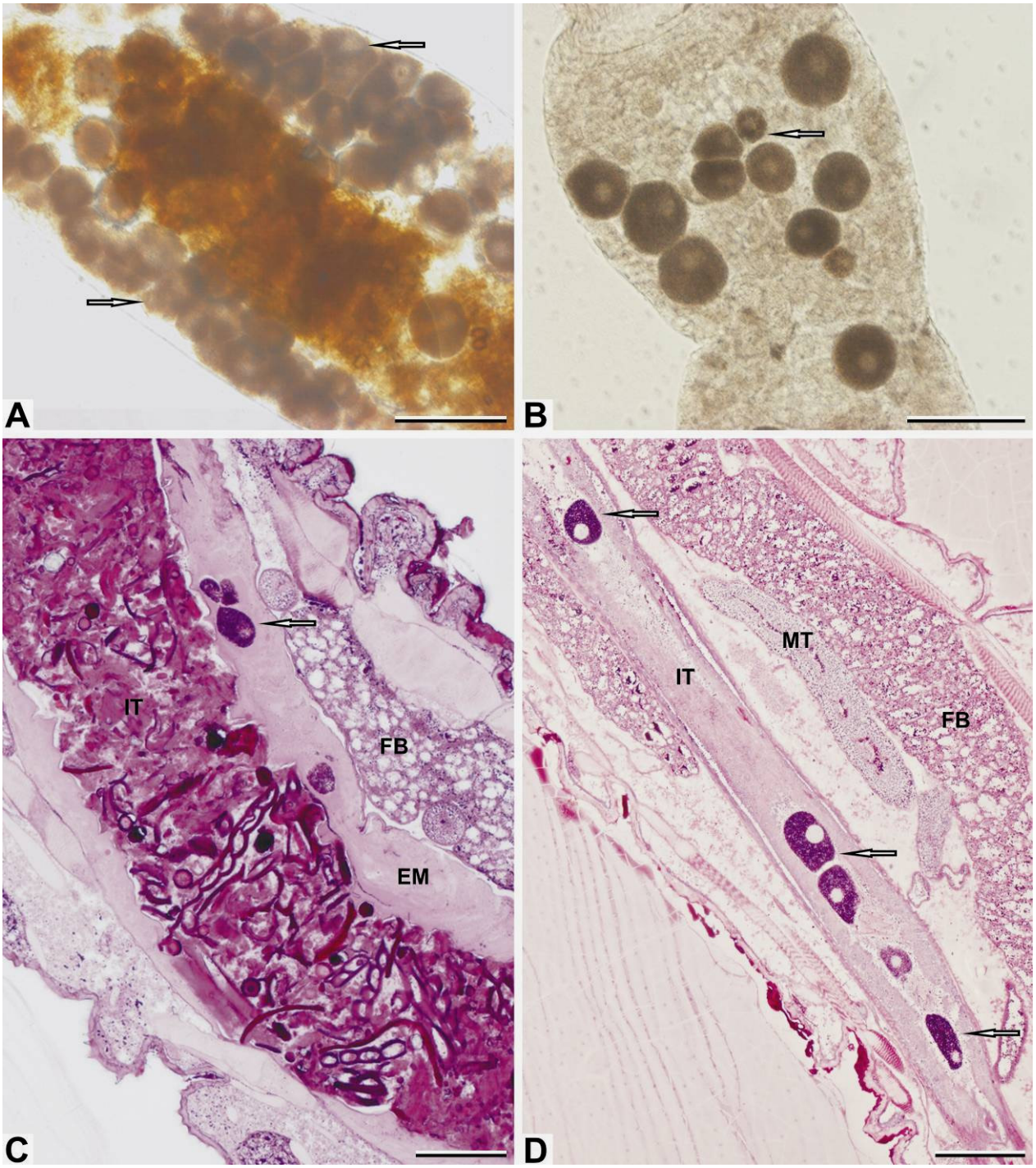


Fig. 4. Histological sections stained with PAS reaction and Ehrlich's acid hematoxylin of *Phlebotomus sergenti* males infected with *Psychodiella sergenti* (A, B) and native preparations of *Ph. sergenti* adults infected with *Ps. sergenti* (C – F).

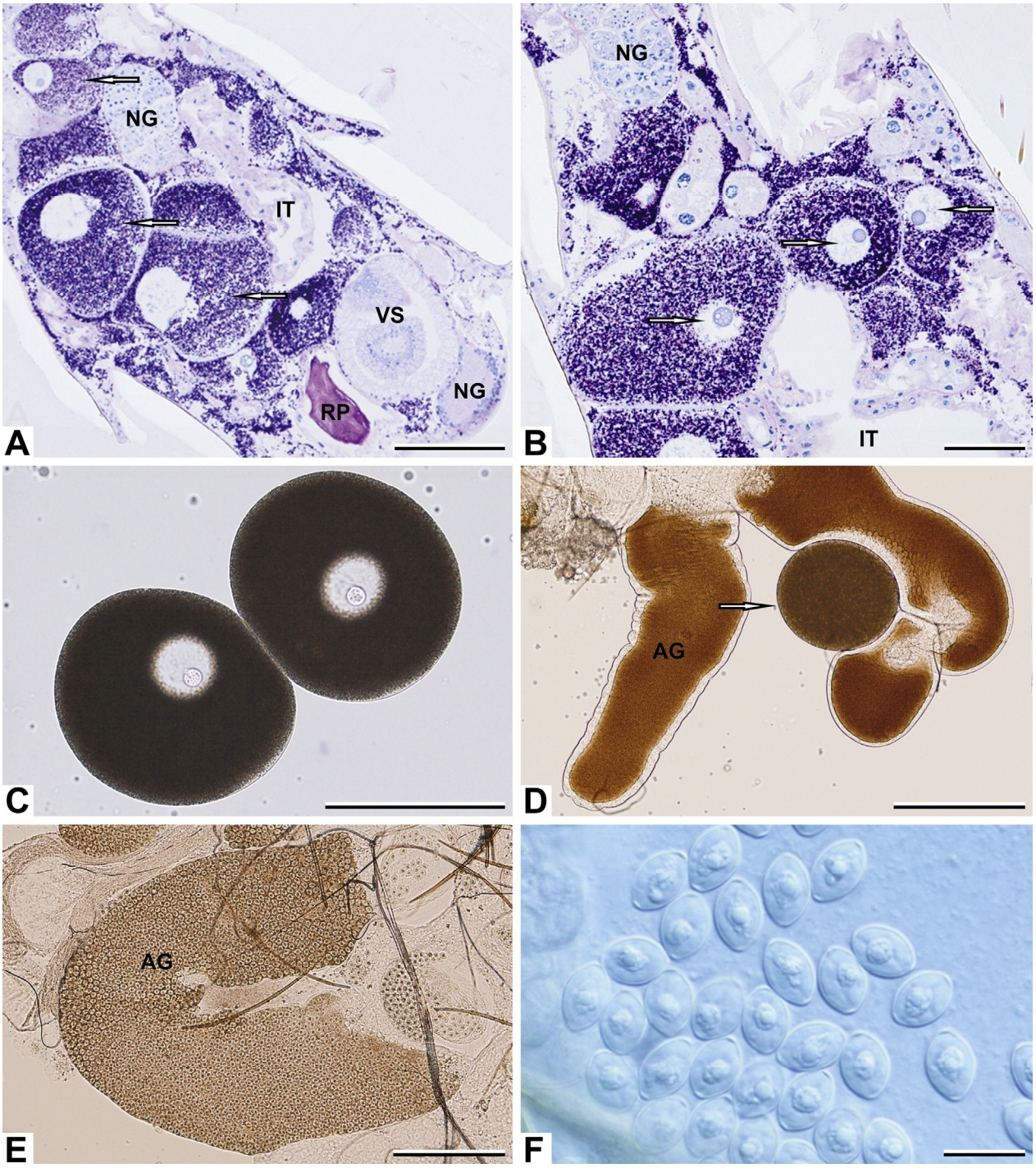
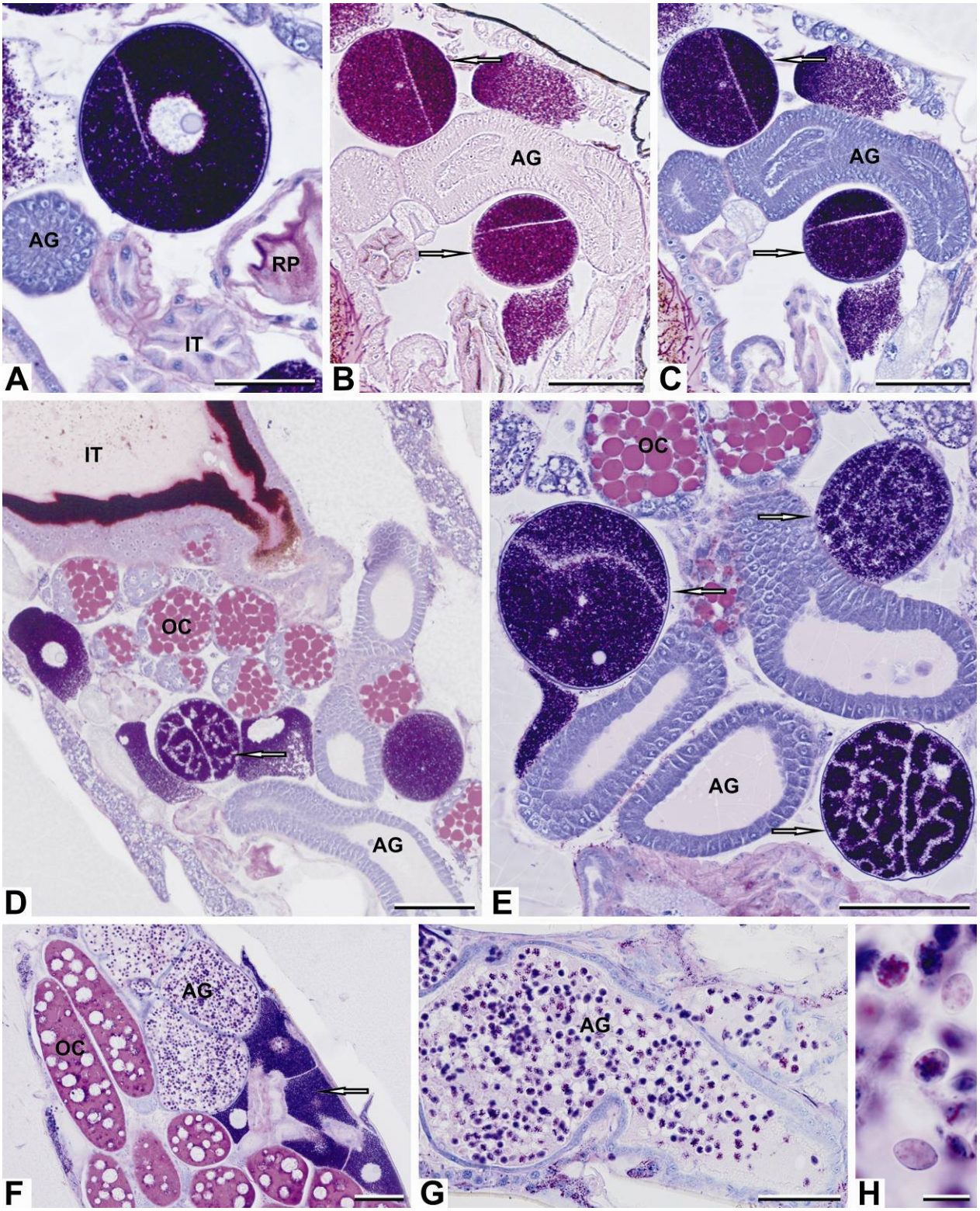


Fig. 5. Histological sections of *Phlebotomus sergenti* females two (A – C), three (D, E) and seven (F – H) days post blood meal infected with *Psychodiella sergenti* stained with PAS reaction and Ehrlich's acid hematoxylin.



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Effects of *Psychodiella sergenti* (Apicomplexa, Eugregarinorida) on Its Natural Host *Phlebotomus sergenti* (Diptera, Psychodidae)

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Abstract

Phlebotomine sand flies (Diptera, Psychodidae) are important vectors of human pathogens. Moreover, they possess monoxenous parasites including gregarines of the genus *Psychodiella* Votypka, Lantova and Volf, which can negatively affect laboratory-reared colonies and have been considered as potential candidates in biological control. In this study, effects of the gregarine *Psychodiella sergenti* Lantova, Volf and Votypka on its natural host *Phlebotomus sergenti* Parrot were evaluated. The gregarines increased the mortality of immature sand fly stages, and this effect was even more apparent when the infected larvae were reared in more dense conditions. Similarly, the gregarines negatively affected the survival of adult males and females. However, no impact was observed on the mortality of blood-fed females, the proportion of females that laid eggs, and the number of eggs oviposited. The ten-time higher infection dose (50 versus 5 gregarine oocysts per one sand fly egg) led to roughly 10 times more gamonts in 4th instar larvae and two or three times more gamonts in females and males, respectively. Our study clearly shows that *Psychodiella sergenti* is harmful to its natural host under laboratory conditions. However, its potential for use in biological control is questionable due to several factors including this parasite's strict host specificity.

Key words

Gregarine, sand fly, mortality, fecundity, infection dose

Introduction

Phlebotomine sand flies are vectors of important human pathogens such as *Leishmania* Ross, *Bartonella* Strong, O'Connor, Winkler and Steigerwalt, and phleboviruses. They are, however, parasitized by various organisms such as viruses, bacteria, fungi, nematodes, mites, and protists including gregarines (reviewed by Warburg et al. 1991). Adult sand flies feed on plant sugars; females need blood to acquire nutrients for egg production. Very little is known about the sand fly breeding sites, in general, eggs are laid to the moist soil, animal burrows, caves, or leaf litter, and four larval instars feed on organic detritus (Lane 1993), sometimes also on dead bodies of adults (Adler and Mayrink 1961). The terrestrial development in dark humid sites facilitates growth and persistence of various sand fly entomopathogens and, at the same time, complicates their collection and examination (Warburg 1991).

Gregarines are parasites of invertebrates, particularly insects. Their effects to hosts vary, and gregarines possessing merogony in their life cycle have even been considered a potential tool for biological control (Perkins et al. 2000). Within the nematoceran Diptera, two gregarine genera have been recently distinguished: the genus *Ascogregarina* Ward, Levine and Craig parasitizing mosquitoes and the genus *Psychodiella* Votypka, Lantova and Volf parasitizing sand flies (Votypka et al. 2009). Neither genera undergoes merogony, and various authors have come to different conclusions concerning their effects to the hosts (Barrett 1968, Walker et al. 1987, Wu and Tesh 1989, Siegel et al. 1992, Sulaiman 1992, Comiskey et al. 1999).

Psychodiella sergenti Lantova, Volf and Votypka is a recently described specific pathogen of *Phlebotomus sergenti* Parrot (Lantova et al. 2010). This sand fly is an important vector of human cutaneous leishmaniasis caused by *Leishmania tropica* (Wright) (reviewed by Jacobson 2003). Newly hatched sand fly larvae become infected by ingesting gregarine oocysts, each of which contains eight sporozoites. The sporozoites then develop in the larval intestine. In adults, the gamonts are found in the hemocoel, and the sexual development of *Ps. sergenti*, i.e. the formation of syzygy followed by creation of gametocysts with oocysts inside, occurs exclusively in blood-fed females. In gravid females (females that took a blood meal and developed oocytes), gametocysts attach to accessory glands, oocysts are injected into the gland lumen, and during oviposition they adhere to the chorion of eggs (Lantova et al. 2010).

Psychodiella gregarines occurred naturally in a colony we had established from *Ph. sergenti* collected in Turkey. After several generations, the colony began to suffer

from high adult mortality, and dissections revealed hemocoels heavily infected by gamonts and gametocysts. To reduce the intensity of infection and increase the fitness of the colony, *Ph. sergenti* eggs were washed by a series of reagents. However, this procedure never completely cleaned the eggs and had to be repeated every generation. The present study, focusing on the effects of *Ps. sergenti* on its natural sand fly host, was made possible only when a new, gregarine-free *Ph. sergenti* colony was established.

Materials and Methods

Sand flies and gregarines. A colony of *Ph. sergenti* free of gregarines was established in 2001 from females originating from Israel (further referred to as IS colony). The infected colony of *Ph. sergenti* used as a source of oocysts was established in 1998 from females originating from Turkey (further referred to as TU colony). The two colonies were kept separately in two insectaries, and maximum care was taken to prevent cross-contamination.

Sand flies were reared at 26°C, in standard conditions used in our laboratory (Volf and Volfova 2011). To sustain the TU colony, it was necessary to reduce gregarine infections by a series of disinfecting solutions described by Poinar and Thomas (1984). Two to five-day-old eggs were washed from the rearing pot with distilled water to filter paper in a Büchner funnel connected to a water pump. First, 70% ethanol was used with the water pump on for 10 to 30 s until all excess liquid was removed. Then, eggs were washed with 5.24% sodium hypochlorite (NaClO) with the pump off for 3.5 min followed by removing all remaining liquid by turning the pump on for 1 min. Subsequently, 10% sodium thiosulfate ($Na_2S_2O_3$) was used for 3.5 min with the pump off. Finally, the eggs were thoroughly washed with distilled water with the pump on and washed down into a new clean pot (Poinar and Thomas 1984).

The process of the collection of gregarine oocysts and experimental infection was described by Lantova et al. (2010). In brief, 30 *Ph. sergenti* females (TU colony) were collected after oviposition and homogenized in 500 µl of phosphate-buffered saline (PBS). This solution was filtered through gauze and centrifuged (1,700 g) for 5 min (MiniSpin, Eppendorf AG, Hamburg, Germany), the supernatant was discarded, the pellet was re-suspended in 200 µl of water, and the number of oocysts was determined using a Bürker counting chamber and an optical microscope (CX31, Olympus Corporation, Tokyo, Japan). The appropriate volume of the oocyst suspension,

corresponding to the required number of oocysts, was then re-suspended in water to a total volume of 1 ml and sprinkled over and mixed with four small heaps of larval food placed in each rearing pot.

Effects of gregarine infection on sand fly mortality and fecundity. To evaluate the effect on immature and adult sand fly stages, two groups of sand flies were established; gravid *Ph. sergenti* females from gregarine-free IS colony were allowed to oviposit, and eggs counted by a stereomicroscope (SZH-ILLD, Olympus Optical Co. Ltd., Tokyo, Japan) were placed into rearing pots using a fine brush. In half of the pots, gregarine oocysts corresponding to an infection dose of 50 oocysts per egg were added to the food of 1st instar larvae. The remaining pots served as control. All rearing pots had the same size and shape, and therefore the total rearing area was the same for the experimental and the control group.

To evaluate the effect on the sand fly immature stages, three experiments were carried out. In the first experiment, we used eight pots with 350 eggs each, four of them were infected with gregarines, and four served as a control. The second experiment evaluated the effect of higher larval density during their development (possible competition for nutrients or space), and five pots had 400 eggs each and the remaining five had 200 eggs each, none of them was experimentally infected. The third experiment evaluated the effect of the gregarine infection together with higher larval density. Five experimental pots had on average 444 eggs that were infected, while each of the five control non-infected pots had on average 257 eggs. In each experiment, all pots were placed into the same rearing box to ensure uniform conditions, and emerging adults from both groups were counted every day. The infection status in the case of the first and the third experiment was determined using an optical microscope after dissection in PBS under a stereomicroscope. The number of emerged adults was compared to the number of eggs.

To evaluate the mortality of adults, two groups of sand flies were established as described above, one non-infected and one infected with a dose of 50 gregarine oocysts per egg. In each group, adults that emerged on the same day were placed in a separate cage. The number of dead males and females and their infection status (determined by dissection under a stereo microscope followed by observation in an optical microscope) were recorded daily. The number of dead adults of both sexes was compared between the infected and the control group.

To assess the gregarine effect on blood-fed females, two groups of control and infected sand flies were established as mentioned above. The mortality of blood-fed females was evaluated after females of the same age from both the control and the experimental group were fed on mice, and the number of dead sand flies and the gregarine infection status were recorded daily. The mortality of the experimental and control group was compared. To assess the gregarine effect on the fecundity, other batches of females (from the control and infected group) that emerged at the same day were fed using an anesthetized mouse, and six days after the blood meal (i.e. one or two days after the defecation of blood meal remains) females were separated into glass tubes. The technique originally used for the establishment of sand fly colonies (Killick-Kendrick and Killick-Kendrick 1991) was adopted. In brief: glass tubes (5 x 1.5 cm) with wet filter paper (4 x 5 cm) inside were closed with gauze and a plastic ring. A small piece of cotton wool with a 50% sucrose solution was provided to females, and all tubes were put into the same rearing box to ensure uniform conditions. The time of laying eggs, the number of eggs, and the gregarine infection of dead females in both groups were recorded daily and compared.

Effects of infection dose on the intensity of infection. Six groups of about 15 gravid females (IS colony) were placed into six pots to oviposit, and the number of eggs was counted under a stereomicroscope. Gregarine oocysts corresponding to infection doses of either 5 or 50 oocysts per egg were added to the food of 1st instar larvae. Each infection dose was used in three rearing pots. All pots were placed in the same rearing box. Larvae and adults of both sexes were dissected at different time intervals. In addition, seven-day-old females were fed on mice and dissected at various days after the blood meal. In males and females without the blood meal, the number of gamonts was compared between the two different infection doses. To evaluate the effect in blood-fed females, the total number of gamonts plus gametocysts was compared between the two different infection doses.

All statistical analyses were performed using STATISTICA 6.0 (StatSoft 2004).

Results

Effects of gregarine infection on sand fly mortality. Gregarine infection reduced the survival of immature sand fly stages as out of 1,400 non-infected eggs emerged 969 adults (69.2%), and out of 1,400 gregarine-exposed eggs emerged 840 adults (60%), this difference was significant ($\chi^2 = 5.59$; df = 1; $P < 0.05$). Moreover,

when the mortality of larvae exposed together to the gregarine infection and more dense rearing conditions was compared to the mortality of non-infected larvae in less dense rearing conditions, the difference was highly significant ($\chi^2 = 31.41$; df = 1; $P < 0.01$); in the infected group, 672 adults emerged from 2,220 eggs (30.3%), while in the control group, 566 adults emerged from 1,286 non-infected eggs (44%). The adult eclosion was recorded daily; in both above mentioned experiments, the gregarine-infected sand flies started to emerge two to four days later than the non-infected sand flies (data not shown). To separately assess the larval survival depending on rearing density, an experiment comparing two groups of non-infected *Ph. sergenti* was carried out. Out of 2,000 eggs, 1,278 (63.9%) adults emerged, and 710 (71%) adults emerged from 1,000 eggs. This difference was not statistically significant ($\chi^2 = 3.01$; df = 1; $P = 0.0828$).

Infected males had significantly higher mortality than the control males (Cox's F-test; $F = 2.2126$; $P < 0.01$) (Fig. 1A). Similarly, infected females without a blood meal had higher mortality than the females in the control group (Cox's F-test; $F = 1.7472$; $P < 0.05$) (Fig. 1B). Conversely, no statistically significant difference was found in the mortality of females after a blood meal (Cox's F-test; $F = 1.2019$; $P = 0.1822$) (Fig. 1C).

Effects of gregarine infection on sand fly fecundity. Two out of 22 infected females did not lay eggs (9.1%), and 14 out of 52 non-infected females did not lay eggs (26.9%), but this difference was not significant (Fisher exact test; $P = 0.1335$). T-tests did not reveal any significant differences in the number of oviposited eggs between infected and control females (Table 1). The average numbers were 50 ± 6 eggs and 42 ± 5 eggs (± SEM) per infected and control female, respectively. When only ovipositing females were included, infected ones laid 55 ± 6 eggs (± SEM), and 57 ± 5 eggs (± SEM) were laid by non-infected females.

Effects of infection dose on the intensity of infection. The infection dose of 50 oocysts per egg resulted in more intense infections than the infection dose of 5 oocysts per egg. In the 4th instar larvae (20 dissected specimens in each group), a ten-time higher infection dose led to roughly 10 times more gamonts (45:458), and the difference between groups was highly significant. In males and females without a blood meal (15 dissected specimens in each group), different infection doses resulted in less pronounced but still highly significant differences in parasite numbers. Adding together the numbers of gamonts in all adult sand flies of the same sex, the difference between the higher and lower infection dose groups was about 1:3 in males (309:994) and less than 1:2 in females (403:757) (Table 2; Fig. 2A).

In blood-fed females (15 dissected specimens in each group), the dose of 50 oocysts per egg led to a significant increase in the total number of gamonts plus gametocysts in comparison to the dose of 5 oocysts per egg. Altogether, this increase was about 1:2.5 (134:314) (Table 2; Fig. 2B).

Discussion

Our results showed that *Ps. sergenti* infection negatively affects the survival of immature sand fly stages. In mosquitoes, higher larval mortality due to infection with *Ascogregarina* species has been recorded e.g. by Barrett (1968) in *Aedes aegypti* (L.) and Garcia et al. (1994) in *Aedes taeniorhynchus* (Wiedemann). Mosquito gregarines develop intracellularly in the larval intestine and have deleterious effect on the midgut cells (Sanders and Poinar 1973). On the other hand, *Ps. sergenti* intracellular stages are not known. Therefore, the main cause of higher mortality of immature sand fly stages could be competition for nutrients and energy between gregarines and a sand fly. This competition may become more important in stressful environmental conditions as was shown in our experiments: the effect on the mortality was more pronounced when the infected larvae were reared in higher density. The higher larval density, when studied separately, did not have significant negative impact on the survival. Similar conclusions were presented in mosquitoes by Comiskey et al. (1999) showing that *Aedes albopictus* (Skuse) larvae and pupae infected with *Ascogregarina taiwanensis* (Lien and Levine) had higher mortality under nutrient-deficient conditions. The joined negative effect of starvation and the gregarine infection was proved also in the black carpet beetle *Attagenus megatoma* (F.): starving eugregarine-infected larvae were losing weight almost twice as rapidly as the non-infected ones (Dunkel and Boush 1969). Similarly, in starving mealworm *Tenebrio molitor* L., the weight loss of eugregarine-infected pupae was larger than in the non-infected ones (Harry 1967).

In adult sand flies, the infection with *Ps. sergenti* significantly decreased the survival of infected males and females without a blood meal (Fig. 1A, B), while the mortality of blood-fed females was not affected (Fig. 1C). This may indicate that more nutritious diet (blood meal) enables infected females to overcome the negative effect of the parasite. Interestingly, sex dependent differences were observed in adult mortality: in males, higher mortality was detected from the second day, whereas all females, regardless of their infection status, survived until the 11th day after emergence from pupa and only started to die thereafter (Fig. 1). This could be advantageous for the

250 gregarine: the longer the female lives, the higher the chance of a blood meal followed
251 by egg production and transmission. There are several studies on the effect of gregarines
252 on the life span of adults. In sand flies, Wu and Tesh (1989) observed increased
253 mortality in adult *Lutzomyia longipalpis* (Lutz and Neiva) infected with *Psychodiella*
254 *chagasi* (Adler and Mayrink). The infected females started to die on the seventh day
255 after emergence and had a higher mortality rate than the control group. In mosquitoes,
256 significant negative effect on the mortality was observed by Garcia et al. (1994) and
257 statistically unsupported higher mortality by McCray et al. (1970) or Beier (1983). The
258 negative effect of the gregarines on the adult mosquito survival could be caused by the
259 gregarine's development in the Malpighian tubules, which severely damages them
260 (Barrett 1968). On the other hand, sand fly gregarines of the genus *Psychodiella*
261 develop in the body cavity of their hosts attached to the oviducts or accessory glands.
262 Histology, however, did not reveal any specific damage to sand fly organs (Lantova,
263 unpublished data).
264 *Psychodiella* gregarines seem to have no effect on sand fly fecundity as in our
265 experiments the infection affected neither the number of ovipositing females nor the
266 number of oviposited eggs. In *L. longipalpis* sand flies infected with *Ps. chagasi*, Wu
267 and Tesh (1989) obtained similar results. In mosquitoes, studies of the effect of
268 *Ascogregarina* on fecundity gave contradictory results; McCray et al. (1970) did not
269 find any significant effect on *Ae. aegypti* infected by *Ascogregarina culicis* (Ross),
270 while Comiskey et al. (1999) reported that parasitized females produced 23% fewer
271 eggs than non-parasitized ones.
272 While the gregarine does not affect blood-fed females, *Leishmania* parasites do,
273 with the effect being significant under stressful conditions (Rogers and Bates 2007).
274 This may raise a question about a possible synergistic effect of both parasites on the
275 sand fly. In nature, however, such co-infections are very rare, mainly because even in
276 leishmaniasis foci the percentage of *Ph. sergenti* carrying *L. tropica* is very low (e.g.
277 Volf et al. 2002, Gebre-Michael et al. 2004).
278 In 4th instar larvae, the ten-time higher infection dose led to roughly 10 times
279 more gamonts, while in adults, the intensity of infection achieved by higher infection
280 dose was only two or three times higher in females and males, respectively (Fig. 2A).
281 We suppose that the number of gregarines that can develop in the host is limited, and
282 the pupal stage is clearly the most critical period for gregarine survival. The effect of the
283 infection dose has not yet been studied in sand fly gregarines, and comparative data are

available only from one mosquito experiment: Reyes-Villanueva et al. (2003) exposed larvae of *Ae. aegypti* and *Ae. albopictus* to various oocyst concentrations of *As. culicis* and *As. taiwanensis* and found linear dependence of infection intensity on the infection dose.

Dissections confirmed our previous observations on the life cycle of *Ps. sergenti* (Lantova et al. 2010), i.e. the gregarines were able to complete the life cycle exclusively in females after a blood meal. Only gamonts were present in males and females without a blood meal. Importantly, it was shown that even the lower dose of 5 oocysts per egg was sufficient for successful infection and completing the life cycle.

In order to determine the number of oocysts commonly produced in one laboratory-reared *Ph. sergenti* female from Turkey, the number of oocysts in females seven days after a blood meal was counted using a Bürker counting chamber. Average number of oocysts per *Ph. sergenti* female was 15,158 ± 1,807 (± SEM). As this sand fly colony produces 32 eggs per female on average (Dvorak et al. 2006), we could estimate the theoretical infection dose in laboratory conditions as 474 ± 56 gregarine oocysts per sand fly egg. However, not all the oocysts are discharged during oviposition; some of them remain in the body carcasses of dead females, which are collected from the breeding pots after oviposition. The experimental dose used in our study (5 or 50 oocysts per egg) is much lower than the one estimated above, but it probably better reflects natural infections in sand fly breeding sites.

Several authors have discussed the possibility of mosquito and sand fly gregarines being useful in biological control with contradictory conclusions; Barrett (1968) and Sulaiman (1992) consider these gregarines useful, while others, e.g. Walker et al. (1987), Wu and Tesh (1989), Siegel et al. (1992), and Tseng (2007) do not. Our experiments showed that *Ps. sergenti* is harmful to its host *Ph. sergenti* under laboratory conditions, and the effects can be influenced by environmental factors. The gregarine's potential for use in biological control is, however, limited by its high host specificity (Lantova et al. 2010) and the lack of knowledge about sand fly breeding sites.

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Table 1. The effect of *Psychodiella sergenti* infection on the number of oviposited eggs by *Phlebotomus sergenti* blood-fed females.

n refers to the number of females

mean, med., SD, min., max. refer to the number of oviposited eggs

CTR non-infected females

INF infected females

Table 2. Statistical comparison of the effects of *Psychodiella sergenti* infection dose (5 and 50 oocysts per egg) on the number of gregarines in *Phlebotomus sergenti*.

In males and unfed females (15 specimens of each stage) only gamonts were found and counted, in blood-fed females (15 specimens of each stage) gamonts and gametocysts were counted and added together.

L4 4th instar larvae (20 specimens)

[a] Numbers represent age of adult sand flies (days after emergence)

[b] Numbers represent days after a blood meal

Table 1. The effect of *Psychodiella sergenti* infection on the number of oviposited eggs by *Phlebotomus sergenti* blood-fed females.

	all females						ovipositing females only					
infection	**n**	**mean**	**med.**	**SD**	**min.**	**max.**	**n**	**mean**	**med.**	**SD**	**min.**	**max.**
CTR	52	41.7	41.0	37.2	0	119	38	57.1	60.0	31.8	1	119
INF	22	50.0	49.5	29.8	0	96	20	55.0	55.5	26.3	4	96
t-val.			–0.9278						0.2476			
df			72						56			
P			0.36						0.81			

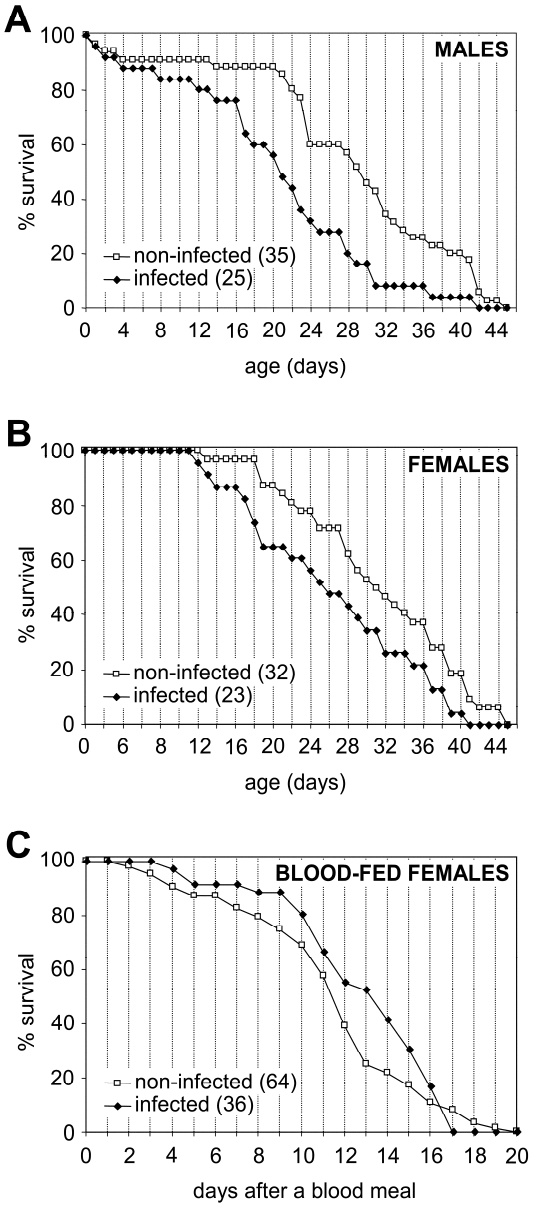
Table 2. Statistical comparison of the effects of *Psychodiella sergenti* infection dose (5 and 50 oocysts per egg) on the number of gregarines in *Phlebotomus sergenti*.

stage		t-value	df	P
L4		–4.5510	38	< 0.01
males[a]	1	–2.8860	28	< 0.01
	4	–2.8426	28	< 0.01
	7	–1.9848	28	0.0570
	9	–2.9449	28	< 0.01
	10	–3.2516	28	< 0.01
	13	–2.8811	28	< 0.01
females[a]	1	–3.3093	28	< 0.01
	4	–0.4474	28	0.6580
	7	–2.9166	28	< 0.01
	9	–2.0672	28	< 0.05
	10	–2.3518	28	< 0.05
	13	–0.2361	28	0.8150
blood-fed females[b]	3	–2.1621	28	< 0.05
	4	–4.1880	28	< 0.01
	7	–1.9899	28	0.0564

Fig. 1. Mortality of *Phlebotomus sergenti* infected with *Psychodiella sergenti* (50 oocysts per egg). (A) males, (B) females without a blood meal, (C) blood-fed females. Numbers in parentheses indicate the number of sand flies.

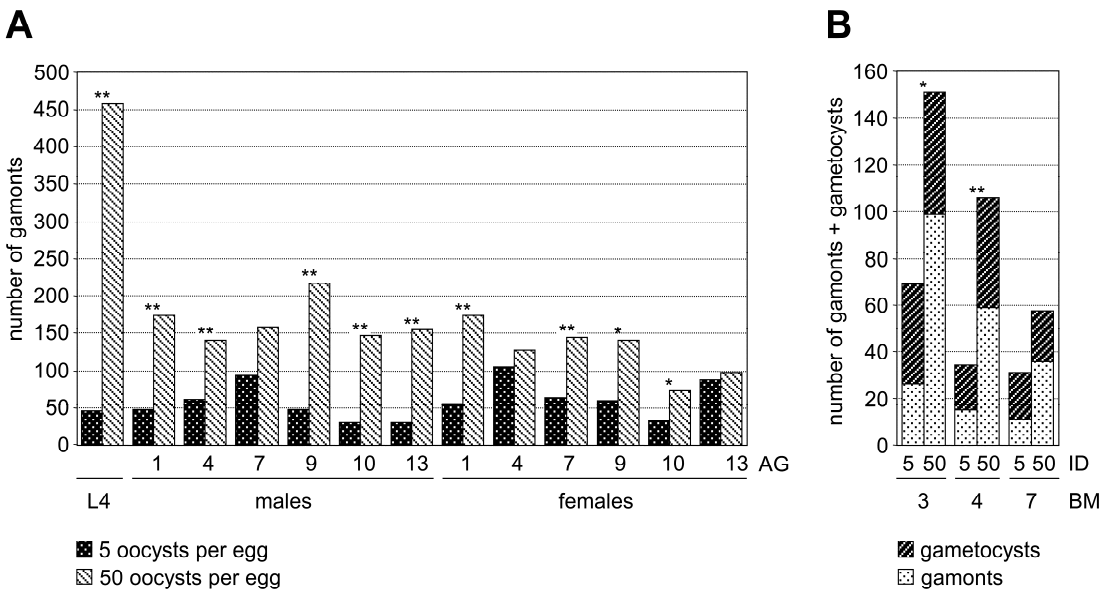
Fig. 2. Effects of gregarine infection dose on the number of *Psychodiella sergenti* individuals in various developmental stages of *Phlebotomus sergenti*. Bars represent: (A) the total number of gamonts in 20 individuals of 4th instar larvae (L4), 15 males, 15 females without a blood meal; (B) the total number of gregarine developmental stages in 15 females blood-fed on mouse. (AG) age of sand flies in days, (ID) infection dose in oocysts per egg, (BM) days after a blood meal. (**) highly significant difference ($P < 0.01$), (*) significant difference ($P < 0.05$) (see Table 2).

437 **Fig. 1.** Mortality of *Phlebotomus sergenti* infected with *Psychodiella sergenti* (50
438 oocysts per egg).



439

Fig. 2. Effects of gregarine infection dose on the number of *Psychodiella sergenti* individuals in various developmental stages of *Phlebotomus sergenti*.



4 SUMMARY OF RESULTS

- Phylogenetic analyses including SSU rDNA sequences of gregarines from *L. longipalpis*, *Ph. sergenti* and *Ph. tobbi* showed that mosquito and sand fly gregarines are closely related to neogregarines; however, they form two disparate monophyletic groups. Within the sand fly gregarine group, *As. chagasi* is divergent from the gregarines from *Ph. sergenti* and *Ph. tobbi*. Based on these molecular features accompanied by biological differences, we divided the former genus *Ascogregarina*, originally comprising of both mosquito and sand fly gregarines, into two: *Ascogregarina* for the mosquito gregarines and a new genus *Psychodiella* Votypka, Lantova and Volf 2009 accommodating the sand fly gregarines.

- We described two new *Psychodiella* species: *Psychodiella sergenti* Lantova, Volf and Votypka 2010 from *Ph. sergenti* and *Psychodiella tobbi* Lantova, Volf and Votypka 2010 from *Ph. tobbi*. These two gregarines differ in the morphology and dimensions of their life cycle stages, particularly in the width and length/width ratio of oocysts. Furthermore, they differ in their life cycle; *Ps. sergenti*, in contrast to other sand fly gregarines, develops sexually only in blood-fed females. *Psychodiella sergenti* and *Ps. tobbi* are strictly host specific; both species were able to fully develop only in their natural sand fly hosts.

- We gave a detailed description of *Ps. sergenti* life cycle using various microscopical methods. Oocysts of *Ps. sergenti* are present on the chorion of eggs, in contact with exochorion sculpturing ridges. Sporozoites, located in the ectoperitrophic space of the 1st instar larval midgut, are never intracellular and have a three-layered pellicle, distinctive mucron and a long conoid. In the 4th instar larvae, the gregarines are located either in the ectoperitrophic space (younger larvae) or in the lumen of the intestine (older larvae). In adult sand flies, the sexual development of the gregarines occurs only in blood-fed females, where the gametocysts attach to the accessory glands and oocysts are injected into their lumen. In males and females fed exclusively on sugar, only gamonts were found.

- During experimental infections, we showed that *Ps. sergenti* has negative impact on its host. It decreases the survival of immature sand fly stages, as fewer adults emerged from gregarine-infected eggs. The mortality of adults was increased by the gregarine infection in males and females without a blood meal; however, blood-fed females were not affected in their mortality, the number of ovipositing females or the number of oviposited eggs. The pupation is clearly a critical point for the gregarine survival, as the number of gregarines in adults is lower than in larvae.

5 CONCLUSIONS

Up until 2009, no gene sequences of any phlebotomine gregarines had been published. For the first time, we successfully sequenced SSU rDNA of three gregarines: *Psychodiella chagasi* from *L. longipalpis* and two new gregarine species from *Ph. sergenti* and *Ph. tobbi.* We showed that both mosquito and sand fly gregarines are closely related to neogregarines. The close relationship of aseptate eugregarines and neogregarines was demonstrated also in other studies (Carreno et al. 1999; Leander et al. 2003a; 2003b; 2006). This may raise the question whether the absence of merogony in some aseptate eugregarines (e.g. the sand fly and mosquito species) is not only the case of the merogony not being detected, as happened e.g. with family Gigaductidae (see chapter 2.2.1). However, merogony has never been reported from sand fly and mosquito gregarines, even thought there is a considerable number of life cycle studies about mostly the mosquito species. Thus, our results show that the usage of solely the life cycle and morphological characteristics in the gregarine taxonomy is not suitable and would benefit from combining both the biological and molecular features, as was shown by Leander et al. (2003b) and Rueckert and Leander (2009).

Because mosquito and sand fly gregarines used to be included in a single genus, it was a great surprise, when they formed distinct groups in our analyses. However, considering their biological features (of the species where the information is available), their paraphyly is supported by several facts. Representatives of these two groups have different hosts: Culicidae for mosquito and Psychodidae for sand fly gregarines. Their life cycles differ considerably; mosquito ascogregarines develop intracellularly in the larval intestine, while this has been reported only for one sand fly species, *Psychodiella mackiei*. In adults, the mosquito ascogregarines develop in the Malpighian tubules and oocysts are defecated, while in sand flies, the gregarines are located in the body cavity, their oocysts are injected into the accessory glands and released during oviposition. Both gregarine groups differ in the morphology of gamonts.

The monophyly of mosquito ascogregarines was recorded in our study as well as by Roychoudhury et al. (2007a). These authors also showed that *As. taiwanensis* and *As. culicis* are closer to each other than to *As. armigerei*, suggesting that this might be connected to the different taxonomic positions of their natural hosts. We observed that gregarines from *Ph. sergenti* and *Ph. tobbi* are closer to each other than to *Ps. chagasi*, which supports this hypothesis, because *Ps. chagasi* parasitize the New World sand

flies, while the other two gregarines are found in geographically and taxonomically different Old World sand flies.

Considering all the biological differences mentioned above, which are well supported by our phylogenetical analyses of SSU rDNA, we divided genus *Ascogregarina* into two, and we established a new genus accommodating sand fly gregarines – *Psychodiella*. The current status of the taxonomy of mosquito and sand fly gregarines is presented in Table 2.

Table 2. Current taxonomical status of mosquito and sand fly gregarines of the former genus *Ascogregarina*.

Ascogregarina Ward, Levine and Craig 1982 mosquito gregarines	***Psychodiella*** Votypka, Lantova and Volf 2009 sand fly gregarines
Ascogregarina culicis (Ross 1898), type species	***Psychodiella mackiei*** (Shortt and Swaminath 1927)
As. tripteroidesi (Bhatia 1938)	***Ps. chagasi*** (Adler and Mayrink 1961), type species
As. barretti (Vavra 1969)	***Ps. saraviae*** (Ostrovska, Warburg and Montoya-Lerma 1990)
As. clarki (Sanders and Poinar 1973)	***Ps. sergenti*** Lantova, Volf and Votypka 2010
As. armigerei (Lien and Levine 1980)	***Ps. tobbi*** Lantova, Volf and Votypka 2010
As. lanyuensis (Lien and Levine 1980)	
As. taiwanensis (Lien and Levine 1980)	
As. geniculati Munstermann and Levine 1983	
As. polynesiensis Levine 1985	

The description of *Ps. sergenti* and *Ps. tobbi* was accomplished, apart from the molecular phylogenetical approach (see above), by evaluating their host specificity and studying and comparing the morphology and size of their life stages and life cycles. Comparisons were accomplished also with *Ps. chagasi*.

Psychodiella sergenti was able to fully develop and complete its life cycle (produce oocysts) only in its natural host *Ph. sergenti*. Oocysts in other than natural host were found only in one blood-fed *Ph. tobbi* female. *Psychodiella tobbi* was rarely able to produce oocysts in *Ph. perniciosus* females, and it almost did not develop in *Ph. sergenti*. Our results are in agreement with Wu and Tesh (1989) suggesting strict host specificity of *Ps. chagasi*. We believe, similarly to these authors, that the gregarines described from a number of sand flies (see chapter 2.3.3) could be new distinct species, although more data is needed to prove this.

Psychodiella sergenti, *Ps. tobbi* and *Ps. chagasi* significantly differ in the size of their life stages, only *Ps. sergenti* and *Ps. tobbi* possess oocysts of equivalent length. However, they significantly differ in the width and length/width ratio, and using these two features, which can be considered unambiguous species characteristics, we can clearly distinguish between *Ps. sergenti* and *Ps. tobbi*, and also differentiate them from *Psychodiella saraviae* and *Ps. mackiei*.

The life cycle of *Ps. sergenti* differs markedly from *Ps. tobbi* and *Ps. chagasi*; in adult *Ph. sergenti*, the sexual development (formation of syzygies, gametocysts and oocysts) occurs exclusively in blood-fed females. In males and unfed females, only gamonts were recorded. Contrastingly, the other two gregarines develop into syzygies, gametocysts and oocysts also in males and unfed females.

Considering all the life cycle and morphological characteristics, accompanied by strict host specificity of these gregarines, we were able to deliver strong evidence that *Ps. sergenti* and *Ps. tobbi* are new species.

In order to characterize in detail the life cycle of *Ps. sergenti* and compare it to other *Psychodiella* species, various microscopical methods were used. *Psychodiella sergenti* oocysts were recorded on the chorion of *Ph. sergenti* eggs for the first time by scanning electron microscopy. The oocysts were often attached to the longitudinal sculpturing ridges, suggesting that the process of the attachment of the oocysts is connected to the formation of exochorion; a viscous fluid is secreted along with the oocysts from the accessory glands, and the consistency of the secretion enables the oocysts to adhere to the egg surface at the site, where drying exochorion produces characteristic sculpturing ridges. Contrastingly to our results, Adler and Mayrink (1961) recorded *Ps. chagasi* oocysts firmly adhered to *L. longipalpis* egg surface at a right angle to the longitudinal axis. These findings suggest that, similarly to the chorion

ornamentation (Nogueira et al. 2004), also the pattern of oocyst attachment might be, to a certain level, species-specific.

Psychodiella sergenti sporozoites were found in the 1st instar larvae always in the ectoperitrophic space of the intestine, sometimes attached to the epithelial cells but never intracellularly. This is in accordance with the life cycle of *Ps. chagasi* (Warburg and Ostrovska 1991) but in contrast to *Ps. mackiei* (Shortt and Swaminath 1927). Sporozoites of *Ps. sergenti* possess a distinctive mucron and a long conoid similar to that of *Ps. chagasi* (Warburg and Ostrovska 1991) but longer than those described in ascogregarines (Sheffield et al. 1971; Chen et al. 1997b); long conoids might be characteristic for *Psychodiella* species. The sporozoites possess a three-layered pellicle. Contrastingly, Vavra (1969), Sheffield et al. (1971) and Sanders and Poinar (1973) in ascogregarines and Warburg and Ostrovska (1991) in *Ps. chagasi* recorded two-layered pellicle. Vavra (1969) points out that such a difference might be due to the fact that the two inner membranes could sometimes be very close giving the impression of a single membrane, or their chemical and structural differences cause them to react differently to the electron microscopy preparation. In the 4th instar larvae, native preparations and histological sections showed gamonts (sometimes syzygies and gametocysts) in the ectoperitrophic space of young individuals. This location protects gregarines from being defecated shortly before pupation, when the larval midgut contents, including the peritrophic matrix, are discharged. After defecation, the gregarines appear in the intestinal lumen.

In adults, the main location of the gregarines is in the body cavity as observed also by Shortt and Swaminath (1927), Adler and Mayrink (1961) and Ostrovska et al. (1990). The gametocysts are attached to the accessory glands as recorded in all sand fly gregarine species (reviewed by Ostrovska et al. 1990). The main distinctive feature of *Ps. sergenti* life cycle is the fact that it develops sexually exclusively in adult females that had a blood meal. The only other sand fly gregarine with similar pattern in its life cycle in adult hosts is an undescribed parasite reported by Ayala (1971) in *L. v. occidentis*. Similarly to Ayala (1973), we hypothesize that the hormonal changes influenced by a blood meal intake trigger the sexual cycle of the gregarine.

There are several features suggesting close relationship between *Ps. sergenti* and its sand fly host and supporting the hypothesis about co-evolution of gregarines and sand flies as mentioned by Ostrovska et al. (1990). (1) The attachment of the oocysts to the exochorion is possibly closely related to the exochorion formation, facilitating the

vertical transmission of the parasite. (2) The gregarine is protected from the expulsion from the larval intestine during pre-pupal defecation by being located in the ectoperitrophic space. This helps the parasite to sustain a certain level of infection during pupation, which was shown to be the most critical for the survivorship of the gregarines (see below). (3) The injection of the oocysts into the accessory gland lumen, facilitated by the host immune response (Warburg and Ostrovska 1989), is a unique mode of vertical transmission. (4) The most remarkable feature is the fact that *Ps. sergenti* does not develop sexually in males or unfed females, which is advantageous for the gregarines, as they only invest energy into the sexual development where the vertical transmission is expected – in blood-fed females.

The close relationship of *Ps. sergenti* and *Ph. sergenti* raises the question, whether or not this parasite has negative impact on its host. In our studies, *Ps. sergenti* infection negatively affects the survival of immature sand fly stages, as fewer adults emerged from the infected eggs than from the control ones. This effect was even more pronounced under stressful conditions provided by rearing the infected larvae in higher density. In mosquitoes, the higher larval mortality due to the infection with *Ascogregarina* species has been recorded by Barrett (1968), Sulaiman (1992) or Comiskey et al. (1999a), and is probably caused by the intracellular development of ascogregarines in the larval intestine, which damages the midgut cells (Kramar 1952; Sanders and Poinar 1973). On the other hand, *Ps. sergenti* intracellular stages have not been recorded. Therefore, the main cause of the higher mortality of pre-imaginal sand flies could be competition for nutrients and energy between the parasite and its host.

Psychodiella sergenti significantly decreases the survival of infected males and females without a blood meal, while the mortality of blood-fed females is not affected. This may indicate that more nutritious diet (blood meal) enables the infected females to overcome the negative effect of the parasite. It is also advantageous for the gregarine, because blood-fed females are the only stages where *Ps. sergenti* develops sexually. Interestingly, unfed females started to die later than males, which gives the gregarine another advantage: the longer the female lives, the higher chance of a blood meal followed by egg production and transmission. The zero effect on blood-fed females is not only on the mortality, but also on their fecundity; the infection did not affect the number of ovipositing females or the number of oviposited eggs. Our results are in

accordance with Wu and Tesh (1989), who showed that *Ps. chagasi* negatively affects adult sand fly mortality, but not fecundity of *L. longipalpis* females.

In the 4th instar larvae, a ten-time higher infection dose led to roughly 10 times more gamonts, while in adults, the intensity of infection achieved by higher infection dose was only two or three times higher. This decrease in the number of gregarines in adult sand flies suggests that the pupal stage is the most critical period for the survival of *Ps. sergenti*. This is also supported by our host specificity infections of *Ps. sergenti* and *Ps. tobbi*, where the number of gregarines was much higher in the larvae than in the adults.

Several authors have discussed the possibility of mosquito and sand fly gregarines being useful in biological control with contradictory conclusions; Barrett (1968) and Sulaiman (1992) consider these gregarines potentially useful, while Walker et al. (1987), Wu and Tesh (1989), Siegel et al. (1992) and Tseng (2007) do not. Our experiments showed that *Ps. sergenti* is harmful to its host *Ph. sergenti*, and the effects can be influenced by environmental factors. This is very important in laboratory colonies, where the infection intensity is usually much higher than in natural conditions, and it has been shown that laboratory-reared colonies can be seriously damaged by the gregarines. However, the potential of *Psychodiella* gregarines for use in biological control is limited by their strict host specificity and the lack of knowledge about sand fly breeding sites (Feliciangeli 2004).

Infection with gregarines activates hemocyte-mediated immune response of sand flies (Warburg and Ostrovska 1989). An interesting subject for further research could be the humoral response of sand flies to *Psychodiella* infection. Insect defensins, members of a large group of antimicrobial peptides (AMP), are mainly active against Gram-positive bacteria (reviewed by Hetru et al. 1998); however, Boulanger et al. (2004) identified a new defensin expressed by *L. major*-challenged *Phlebotomus duboscqi,* whose recombinant form has antiparasitic activity against this parasite. It would be interesting to study the effect of *Psychodiella* infection on the defensin expression in sand flies, and because co-infections of sand flies with *Psychodiella* and *Leishmania* are possible, it would also be interesting to evaluate the effect of *Psychodiella* on the vector competence of sand flies for *Leishmania*.

Furthermore, the lack of molecular phylogenetic studies calls for sequencing various genes of more *Ascogregarina* and *Psychodiella* species. There is clearly a

hidden biodiversity among sand fly gregarines and more species are expected to be described from different sand flies in the future. For example, we recently sequenced gene for SSU rRNA of a gregarine found in *Sergentomyia christophersoni*, and according to the sequence, it is different from *Ps. chagasi*, *Ps. sergenti* or *Ps. tobbi*.

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